12-2011

# ARSENIC REPRESSES MYOGENESIS AND NEUROGENESIS THROUGH EPIGENETIC MECHANISMS, REPRESSED TRANSCRIPTION FACTORS, AND ALTERED WNT SIGNALING PATHWAY

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A Dissertation
Presented to
the Graduate School of
Clemson University

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In Partial Fulfillment
of the Requirements for the Degree
Doctor of Philosophy
Environmental Toxicology

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by
Gia-Ming Hong
December 2011

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Accepted by:
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# ABSTRACT

Arsenic is a toxicant commonly found in water systems around the world. Evidence from epidemiological studies indicates that chronic arsenic exposure can result in cancer, central nervous system and sensory deficits, effects on development, and neuromuscular deficits. However, the molecular mechanism of arsenic's toxicity remains largely unclear. In this study, both C2C12 mouse myoblast cells and mouse embryonic stem cells (mESCs) were used as models of arsenic mediated developmental toxicity in humans to investigate the effects of sodium arsenite on cellular differentiation.

Results from our first and second studies indicate that exposure of 20nM sodium arsenite to C2C12 mouse myocyte cells results in delayed muscle differentiation due to a reduction myogenin expression. The repressed myogenin expression was due to a combination of abnormal DNA methylation and histone modifications on the myogenin promoter, repressed Igf-1 and Mef2 expression, and enhanced Ezh2 expression. In the third study, we examined arsenic's effects earlier in development, using mouse P19 embryonic carcinoma cells as our model. These results indicate that arsenic inhibits myogenesis and neurogenesis due to the reduction of essential myogenic and neurogenic transcription factors, such as Pax3, Myf5, MyoD, myogenin, neurogenin 1&2, and NeuroD. The reduction of these transcription factors was, in part, due to repressed Wnt/β-catenin signaling during early embryogenesis following arsenic exposure. In conclusion, these results illustrate the mechanisms of how environmental realistic arsenic exposure impacts development. More importantly, this study gives us reasonable motivation to ask whether the

drinking water standard for arsenic is protective of fetal health.

# DEDICATION

I dedicate this work to my family, my wife, and my son.

# ACKNOWLEDGMENTS

I would like to express my deepest appreciation to my advisor, Dr. Lisa Bain, for her positive attitude, encouragement, and support throughout my PhD studies. Without her intelligent advice and tireless guidance, I would never have accomplished this work. I would also like to thank my committee members, Dr. William Baldwin, Dr. Susan Chapman, and Dr. Charles Rice, for their critical comments and precious suggestions.

I appreciate Dr. Terry Bruce from the Clemson University Imaging Facility and Ms. Nancy Korn from Clemson University Histology Core Lab for their technical assistance. I am grateful for my lab-mates and other friends at Clemson for their help and support. My special thanks go to Dr. Kristen Gaworecki, Dr. Linda Mota, Dr. Basma Damiri, Ben Green, Amanda Steffens, Yangchun, and Gautam for their continuous encouragement through these past three and a half years.

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CHAPTER ONE

# INTRODUCTION

## BACKGROUND

Arsenic is a naturally occurring mineral found in soil, bedrock, and water. It exists mainly in four valency states (-3, 0, +3, and +5), depending upon environmental conditions. The trivalent arsenic ($As^{+3}$) and the pentavalent arsenate ($As^{+5}$) are widely present in natural waters and are soluble over a wide range of pH conditions (Jones, 2007). The primary route of exposure is through drinking water. Once ingested, inorganic arsenic is easily absorbed from the gastrointestinal tract, distributed throughout the body, often metabolized by methylation, and excreted primarily in urine (Benbrahim-Tallaa and Waalkes, 2007; Cohen et al., 2006; Reichard and Puga, 2010).

Chronic arsenic poisoning is a global health problem affecting millions of people (McDonald et al., 2007; Medrano et al., 2010; Wang et al., 2009). Evidence from epidemiological studies indicate that chronic arsenic exposure in drinking water can result in cancer, central nervous system and sensory deficits, effects on development, and neuromuscular deficits (Andrew et al., 2007; Benbrahim-Tallaa and Waalkes, 2007; Kozul et al., 2009; Mohammad et al., 2009). Unfortunately, the mechanisms responsible these multiple adverse outcomes remain largely unclear and likely are multi-factorial. With increasing evidence showing that low arsenic concentrations increase cancer risk (Cohen et al., 2006), the WHO lowered the recommendation level to 10 ppb in 1993 (WHO, 1993), whereaseas, the U.S. EPA reduced its drinking-water standard in regulated public water sources from 50 ppb to 10 ppb (0.67–0.13 μM) in 2006 (EPA, 2001).

## ARSENIC METABOLISM

Arsenic is primary metabolized in the liver, where arsenate ($As^{+5}$) is bio-transformed to arsenite ($As^{+3}$) by arsenate reductase (EC 1.20.4.1). Arsenite ($As^{+3}$) is then sequentially methylated to form methylarsonate ($MMA^{+5}$) and dimethylarsinic acid ($DMA^{+5}$) by arsenic methyltransferase (As3MT) or arsenite methyltransferase using *S*-adenosylmethioine (SAM) as a methyl group donor (Fiskus et al., 2009; Reichard and Puga, 2010; M. Vahter, 2009). Due to the low toxicity of pentavalent methylated species, formation of methylated metabolites of inorganic arsenic has been regarded as a detoxification process for many years (Gebel, 2002; Zhou et al., 2008). However, recent findings of a high toxicity of trivalent methylated species have suggested that intermediates and products formed in this methylation pathway may be more reactive and toxic than inorganic arsenic (Cohen et al., 2006; Thomas et al., 2010; Tseng, 2009; Zhou et al., 2008).

## ARSENIC EXPOSURE RESULTS IN ALTERED DNA METHYLATION

Recently, more attention has been focused on arsenic-induced DNA methylation. In many cells, arsenic undergoes enzymatic mono- and dimethylation, which is mediated by arsenic methyltransferase (As3MT) using *S*-adenosylmethioine (SAM) as a methyl group donor (Drobna et al., 2009; Jones, 2007; Reichard and Puga, 2010; Thomas et al., 2010). However, SAM is also known as the universal methyl donor for the majority of methyltransferases that modify DNA, RNA, histones and other proteins, dictating replicational, transcriptional and translational fidelity, mismatch repair, chromatin modeling, epigenetic modifications and imprinting (Loenen, 2006). Since the

SAM/methyltransferase pathway for biotransformation of arsenic overlaps with the DNA methylation pathway, in which donation of methyl groups from SAM to cytosine produces 5-methylcytosine in DNA, one could hypothesize that the arsenic-induced adverse outcomes may be due to the imbalanced SAM pool within the organism. Additionally, aberrant induction of methyltransferases could lead to abnormal epigenetic controls on gene expression. To this end, studies have been conducted to show that overexpression of As3MT alters the transcriptional profiles upon arsenic exposure in human urothelial cells (Hester et al., 2009). Results from this microarray study indicate that "regulation of transcription" is the top 3$^{rd}$ most significant pathway. There were five significantly expressed genes in this pathway, all of which belong to heterochromatin protein 1 family in transcriptional silencing (Hester et al., 2009).

In addition, Zhao and coworkers have shown that arsenic-induced global DNA hypomethylation occurred concurrently with aberrant gene expression and in the presence of reduced levels of SAM in nude mice (Zhao et al., 1997a). Moreover, Ramírez and coworkers have demonstrated that addition of SAM rescues the arsenic-induced alterations oin microtubule polymerization in HeLa cells (Ramírez et al., 2008). Individuals chronically exposed to arsenic in their drinking water had hypermethylation of the p53 and p16 promoters (Chanda et al., 2006), whereas whole genome hypermethylation in persons exposed to different levels of arsenic in drinking water has also been reported (Majumdar et al., 2009). Collectively, several cell culture and animal studies indicate that arsenic exposure results in both DNA hypomethylation and

hypermethylation, and thereby leading to aberrant gene expression (Reichard and Puga, 2010; Salnikow and Zhitkovich, 2008).

DNA methylation not only regulates gene expression, but also plays a role in muscle development. For example, Hu and coworkers have demonstrated that methylation of the α-smooth muscle actin (α-SMA) promoter inhibits α-SMA expression in mouse myofibroblast differentiation. The reduced α-SMA expression was due to the methylated CpGs block the binding of the essential transcription factor, transforming growth factor β (TGFβ), to its promoter regions (Hu et al., 2010). Nakatsuka *et al.* found that global DNA demethylation induces skeletal myogeneic differentiation of mouse dental pulp stem cells by up-regulating myogenin, MyoD1, and myosin heavy chain expression (Nakatsuka et al., 2010). This information suggests that arsenic-mediated DNA methylation could alter muscle development and myotube formation by altering muscle-specific transcription factors.

**ARSENIC EXPOSURE ALTERS HISTONE MARKS**

Arsenic exposure can modify histone acetylation and methylation patterns, leading to changes in gene expression. For example, arsenic-triggered phosphoacetylation of histone H3 in the c-fos and c-jun promoters have been identified in human diploid fibroblasts (Li, 2003). H3K9 dimethylation (H3K9me2) and H3K9 trimethylation (H3K9me3), both of which represent gene silencing marks, were induced at a global level in human lung carcinoma A549 cells and in normal human bronchial epithelial BEAS-2B cells upon exposure to low levels (0.1 μM) of arsenic (Zhou et al., 2008). Similarly, H3K4 trimethylation (H3K4 me3), which represents transcriptional activation, was

significantly induced at a global level in A549 cells and BEAS-2B cells upon exposure to 0.1μM arsenic for 7 days. Interestingly, this epigenetic mark still remained elevated and inherited through cell division for 7 days following removal of arsenic (Zhou et al., 2009). These two reports conclude that arsenic may induce histone remodeling to form either active or repressive chromatins within the same cell, indicating that arsenic plays a role in epigenetic control of gene expression.

In addition to histone modification at a global level, arsenic-induced histone modification occurs in promoter regions of specific genes. For example, arsenic can inhibit transcription mediated by all five steroid receptors (SRs) and two related class II receptors, the retinoic acid (RA) receptor and the thyroid hormone (TH) receptor, at environmentally relevant arsenic concentrations in cell culture and in animal models (Bodwell et al., 2006; Davey et al., 2007; Davey et al., 2008). The repressed steroid receptor-regulated gene transcription was due to the decrease in H3K18ac and H3R17me at glucocorticoid receptor-regulated promoters after treatment with arsenic (Barr et al., 2009). These studies suggest that arsenic can act not only as a potent endocrine disruptor, but also a transcription factor that regulates histone remodeling. Moreover, in human UROtsa cells, arsenic induces Wnt5A transcription by enriching the permissive chromatin marks, H3K14ac and H3K4me2, and repressing the repressive chromatin marks, H3K27me3 and H3K9me2, in the Wnt5A promoter region (Jensen et al., 2009). Given this information, arsenic's mechanism of action may also be mediated by epigenetic changes that lead to aberrant gene expression.

## ARSENIC'S EFFECTS ON MUSCLE DEVELOPMENT

In addition to the cancer endpoints, arsenic is also known as a developmental toxicant. In humans, arsenic can pass through the placental barrier, resulting in adverse developmental effects during pregnancy (Agusa et al., 2010; Concha et al., 1998; Markowski et al., 2011; Raqib et al., 2009; von Ehrenstein et al., 2006). Evidence from epidemiological studies indicates that arsenic exposure increases the risk of negative pregnancy outcomes (Table 1).

**Table 1.1: Epidemiological studies linking arsenic and adverse pregnancy outcomes**

| As concentration | Risk of adverse outcome | Country (reference) |
|---|---|---|
| 276-408 ppb | 1.4X neonatal death increase | Bangladesh (Rahan et al., 2007) |
| >200 ppb | 2.8X neonatal death increase | India (von Ehrenstein et al., 2006) |
| 200 ppb | 6X stillbirth increase | India (von Ehrenstein et al., 2006) |
| 600 ppb | 2X stillbirth increase | China (Sen and Chaudhuri, 2008) |
| >90 ppb | -30g birth weight reduction | Taiwan (Yang et al., 2003) |
| 40 ppb | -57g birth weight reduction | Chile (Hopenhayn et al., 2003) |

For example, studies in India find a 2.8-fold increased risk of neonatal death and a 6-fold increased risk of stillbirth in women exposed to $\geq$ 200 ppb of arsenic through drinking water compared to those that drank uncontaminated water (von Ehrenstein et al., 2006). In Taiwan and Chile, average birth weight was lower in regions with increased arsenic in drinking water (Hopenhayn et al., 2003; Yang et al., 2003). Even an outcome

as minor as lowered birth weight can have physiological consequences later in life. For example, low-birth-weight piglets form reduced numbers of muscle fibers during prenatal myogenesis, and therefore, these animals do not exhibit postnatal "catch-up " growth (Rehfeldt and Kuhn, 2006). Arsenic's effects on muscle differentiation may play an important role and result in adverse developmental outcomes.

To date, results from *in vitro* and *in vivo* studies have shown that arsenic-exposure has adverse effects on muscle differentiation. In fish models, the offspring of mummichogs (*Fundulus heteroclitus*) whose parents were exposed to arsenic displayed curved/stunted tails, which was correlated to the induction of myosin light chain, type II keratin, tropomyosin, and parvalbumin in the hatchlings (Gonzalez et al., 2006). Zebrafish embryos exposed to arsenic, albeit at high concentrations, had increased dorsal curvature and flat heads, and reduced heart rates. The morphological malformations in the heart were due to the reduced myosin heavy chains protein expression in the ventricle and atrium (Li et al., 2009). In mammalian systems, arsenic exposure to mouse C2C12 myoblasts resulted in delayed muscle differentiation into myotubes (Steffens et al., 2011). Rodent models indicate that arsenic suppresses the regeneration of injured muscles (Yen et al., 2010), and disrupts the smooth muscle integrity around the blood vessels in the thoracic aorta (Lim et al., 2011). In addition, mice exposed to arsenic *in utero* had increased smooth muscle actin protein expression in their lungs, which altered pulmonary structure and function (Lantz et al., 2009). Collectively, these results suggest that arsenic acts as a developmental toxicant by affecting the development of the musculature.

However, the molecular mechanisms behind the arsenic-mediated adverse effects on muscle development remain largely unknown.

Skeletal muscle development in vertebrates occurs in several defined steps. Mesoderm-derived precursor cells become committed to the myogenic lineage and develop into myoblasts, which subsequently fuse to form myotubes. Later, myotubes mature into multinucleated highly specialized muscle fibres that show cross striation (Brandsaberi, 2005; Darabi and Perlingeiro, 2008). During myogenesis, several myogenic transcription factors are required to regulate muscle differentiation at different stages. For example, Pax3 is needed so that embryonic progenitor cells can enter into the myogenic program (Messina and Cossu, 2009; Otto et al., 2006; Ridgeway and Skerjanc, 2001). Expression of muscle regulatory factors (MRFs), such as Myf5, Mrf4, and MyoD, are required for myoblast determination, while myogenin is needed for terminal differentiation (Carvajal and Rigby, 2010; Gianakopoulos et al., 2011; Yokoyama and Asahara, 2011). Aside from muscle regulatory factors, other molecules also regulate the development of skeletal muscle. For example, myocyte enhancer factor 2 (MEF2) can promote muscle differentiation by directly up-regulating myogenin expression (Lu et al., 2000; Molkentin et al., 1995; Ohkawa et al., 2006). Signaling molecules, such as insulin-like growth factor 1 (IGF-1) and myostatin regulate myogenin expression *via* the PI3K/AKT pathway during skeletal muscle development (Alzhanov et al., 2010; Artaza et al., 2002; Yang et al., 2007). Moreover, other chromatin-modifying enzymes, such as the polycomb Ezh2 methyltransferase (Ezh2) and G9a histone methyltransferase, have been reported to play a role in the repression of muscle differentiation by epigenetically

repressing myogenic transcription factors (Caretti et al., 2004; Davis et al., 2006; Juanet al., 2009). Given this information, whether arsenic could alter these muscle-specific regulatory factors and thereby leading to the reduced muscle differentiation will be examined in this study.

## ARSENIC'S EFFECTS ON NEURONAL DIFFERENTIATION

Arsenic can cross the blood-brain barrier (Au et al., 2008; Jin et al., 2006; Kiguchi et al., 2010; Knipp et al., 2007; Xi et al., 2010) and accumulate in the developing brain, thereby leading to neurotoxicological effects on the developing nervous system. In 1955, a mass poisoning due to arsenic contaminated infant formula in Japan caused 130 infant deaths out of 12,000 suspected victims (Hamamoto, 1955; Koyama, 1955). The follow-up study on more than 600 surviving victims indicates that these survivors, now in theirs 50s, have suffered from mental retardation and neurological diseases (Dakeishi et al., 2006). In addition, other epidemiological studies have shown that arsenic exposure during early life stages correlates with adverse neurotoxicological effects (Table 2). For instance, arsenic contamination in drinking water has been linked to neurological diseases, such as hearing losses, mental retardation, refraction anomaly, and lower intelligence quotient score (Bencko et al., 1977; Calderon et al., 2001; Tsai et al., 2003).

**Table 1.2: Epidemiological studies linking arsenic and adverse neurotoxicological effects**

| As concentration | Risk of adverse outcome | Country (reference) |
|---|---|---|
| 900 ppb | Hearing losses | Czech Republic (Bencko and Symon, 1997) |
| 540-590 ppb | Refraction anomaly, hearing losses, and IQ <85 | Japan (Dakeishi et al., 2006) |
| 130 ppb | Altered pattern memory and switching attention | Taiwan (Tsai et al., 2003) |
| 40 ppb | Lower full-scale and verbal IQ | Mexico (Calderon et al., 2001) |
| 20 ppb | Lower full-scale and verbal IQ | USA (Wright et al., 2006) |

Indeed, arsenic exposure can repress neuronal differentiation. For example, arsenic exposure induces apoptosis in primary cultures of rat cerebellar neurons through the induced nuclear fragmentation and condensation in cerebellar neurons (Namgung and Xia, 2001). In neuroblastoma cells, arsenic exposure inhibits neuronal proliferation, differentiation, and neurite outgrowth (Cheung et al., 2007; Wang et al., 2010). Additionally, Piao *et al.* have observed that arsenic exposure in drinking water induces several histopathological changes in the mouse brain, including nuclear vacuolation, and neurite loss in the cerebral and cerebellum cortex (Piao et al., 2005). Moreover, rats exposed *in utero* to arsenic via the dams' drinking water showed the loss of activity in the locomotion and in the movement of limbs (Chattopadhyay et al., 2002; Rodríguez et al., 2002). Taken together, these studies indicate that arsenic exposure also has adverse effects on neuronal development.

Neuronal development and regulation differs depending on the specific neuronal subpopulation. In sensory neuron lineage, for example, the Wnt/ β-catenin signaling pathway commits neuronal crest-derived progenitor cells to sensory neural precursors (Howard, 2005). Pax3, neurogenin1, and neurogenin 2 then are required for neuroblast determination (Cau et al., 2002; Kim et al., 2011; Ma et al., 1999). Later, the expression of NeuroD, which is regulated by neurogenin 1 and neurogenin 2, promotes the terminal differentiation of sensory neurons (Cherry et al., 2011; Howard, 2005; Ohsawa and Kageyama, 2008). Therefore, whether arsenic exposure alters the expression of neuron-specific transcription factors will be examined in this study.

**EMBRYOTOXICITY OF ARSENIC**

Laboratory studies have suggested negative effects of arsenic on early embryonic development. For example, zebrafish embryos exposed to 0.5mM arsenic displayed reduced survival, delayed hatching, and retarded growth (Li et al., 2009). In rodents, arsenic exposure caused total embryo lethality, major deformities, complete failure to undergo zona lysis, and significantly higher number of blastomere cells with fragmented DNA in embryos at concentrations of 500 to 750 nM (Unis et al., 2009). In addition, Petrick and coworkers have shown *in utero* arsenic exposure through maternal drinking water causes altered gene and protein expression, such as β-catenin, GSK3 beta, Tcf /Lef, integrin b3, and interleukin-4 in the developing lung. The reporter analysis indicates that β-catenin signaling is a likely site of action (Petrick et al., 2009). *In utero* arsenic exposure also has transplacental carcinogenic effects on newborn mice. For example, male offspring exposed to 85 ppm arsenic *in utero* had increases in adrenal cortical

adenoma and hepatocellular carcinoma incidence during adulthood, while female offspring developed lung carcinoma and ovarian tumors (Waalkes et al., 2007). Moreover, several other studies have observed alterations in DNA methylation and a complex set of aberrant gene expression in newborn mice whose parents were exposed to 85ppm arsenic during pregnancy (Xie et al., 2007).

To date, embryonic stem cells are one of the most promising models for the evaluation of developmental toxicity (Adler et al., 2008; Bal-Price et al., 2010) because they are capable of differentiating into every specialized cell type (Angello et al., 1997; Vanderheyden and Defize, 2003; Wobus and Guan, 1998). To this end, results from embryotoxicity hazard assessment, using mouse stem cells, classified arsenic as an embryotoxic metal with an $IC_{50}$ ranging from 0.7 to 1.3μM (Stummann et al., 2008). In human stem cells, Flora and Mehta have demonstrated that 76nM arsenic not only altered the pluripotency of stem cells but also caused a significant down regulation of genes indicative of all the three germ layers (Flora and Mehta, 2009). For instance, the expression of GATA-4, which is an indicative marker of cardiac muscle in endoderm lineage, was significantly repressed upon exposure to 1ppm arsenic (Flora and Mehta, 2009). Moreover, Tokar and coworkers have demonstrated that 5μM arsenic directly transforms normal human prostate epithelial stem cells into an aggressive pluripotent cancer stem cell phenotype (Tokar et al., 2010). Collectively, these and other results suggest that arsenic could act as a developmental toxicant and affect the development of the reproductive system, nervous system, and skeletal system.

## WNT/ β -CATENIN SIGNALING IN EMBRYOGENESIS

Wnt molecules are cysteine-rich secreted glycoproteins that can locally interact with the Frizzled (FZD) family of receptors on the plasma membrane. Wnt signaling can be classified as canonical (β-catenin dependent) or non-canonical (β-catenin independent) (Geetha-Loganathan et al., 2008; Ling et al., 2009; Liu et al., 2008). In silenced canonical Wnt signaling, β-catenin proteins are degraded through proteolysis in cytosol in the absence of Wnt ligands. When cells receive Wnt signals, the Wnt molecule can bind to a frizzled (Fz) receptor to activate canonical Wnt signaling. This then inhibits β-catenin degradation and stabilizes β-catenin in the cytosol. The accumulated cytoplasmic β-catenin can be subsequently translocated into nucleus, where it forms a complex with Tcf/Lcf transcription factors to initiate the transcription of Wnt-target genes (Geetha-Loganathan et al., 2008; MacDonald et al., 2009; Novak and Dedhar, 1999).

During early vertebrate development, the canonical Wnt/ β-catenin signaling pathway plays an important role in developmental process, such as somite formation and neural crest development (Borello et al., 2006; Burstyn-Cohen et al., 2004; Geetha-Loganathan et al., 2008; Huelsken and Birchmeier, 2001; Schmidt et al., 2008). In embryonic stem cells, β-catenin regulates self-renewal and cell fate decisions (Ling et al., 2009; Liu et al., 2008; Lyashenko et al., 2011). For instance, Lyashenko *et al.* have used a β-catenin-deficient mouse stem cell line to demonstrate that self-renewal is maintained in the absence of β-catenin, whereas wild-type stem cells form all three germ layer (Lyashenko et al., 2011). It has also been shown that β-catenin can trigger cells to differentiate into a myocyte lineage in P19 mouse embryonal carcinoma cells

(Petropoulos and Skerjanc, 2002) and cause neuronal differentiation in the WWE6 ES cells (Otero et al., 2004) without any chemical treatments. Therefore, these studies suggest that β-catenin is required for early embryonic development. To this end, it is worthy to understand the effects of arsenic on Wnt/ β-catenin signaling using stem cell models. Altered Wnt/ β-catenin signaling due to arsenic-exposure may provide insight into the mechanisms behind its toxicity in earlier developmental stages.

**OBJECTIVES OF THIS STUDY**

Our preliminary results indicate that 20 nM arsenic delays muscle cells differentiation and suppresses the expression of myogenin. However, the molecular mechanisms of arsenic's toxicity remain largely unknown. Therefore, the goal of this study was to investigate the effects of sodium arsenite on cellular differentiation using both C2C12 mouse myoblast cells and mouse embryonic stem cells (mESCs) as models. The specific objectives include:

1. to determine the epigenetic mechanisms by which arsenic exposure alters the expression of myogenin
2. to identify potential regulatory mechanisms that regulate myogenin expression upon arsenic exposure
3. to investigate whether the arsenic exposure delays the differentiation of mouse embryonic stem cells (mESCs) into muscles and neurons through alterations in the Wnt/ β-catenin signaling pathway

Accomplishing these objectives should provide insight into how arsenic represses muscle differentiation through abnormal epigenetic mechanisms and changes in transcription factor expression. Additionally, arsenic's effects on the Wnt/ β-catenin signaling pathway during early embryogenesis and its consequences for cellular differentiation will be determined using a mouse stem cell model.

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# CHAPTER TWO

# SODIUM ARSENITE DELAYS THE DIFFERENTIATION OF C2C12 MOUSE MYOBLAST CELLS THROUGH ALTERED METHYLATION PATTERNS ON THE TRANSCRIPTION FACTOR MYOGENIN

This manuscript was published in *Toxicology and Applied Pharmacology* and is in the format required of that journal. The citation is:
Amanda A. Steffens, Gia-Ming Hong, and Lisa J. Bain, "Sodium arsenite delays the differentiation of C2C12 mouse myoblast cells and alters methylation patterns on the transcription factor myogenin", *Toxicology and Applied Pharmacology* 250 (2):154-161, 2011

## Abstract

Epidemiological studies have correlated arsenic exposure with cancer, skin diseases, and adverse developmental outcomes such as spontaneous abortions, neonatal mortality, low birth weight, and delays in the use of musculature. The current study used C2C12 mouse myoblast cells to examine whether low concentrations of arsenic could alter their differentiation into myotubes, indicating that arsenic can act as a developmental toxicant. Myoblast cells were exposed to 20nM sodium arsenite, allowed to differentiate into myotubes, and expression of the muscle-specific transcription factor myogenin, along with the expression of tropomyosin, suppressor of cytokine signaling 3 (Socs3), prostaglandin I2 synthesis (Ptgis), and myocyte enhancer 2 (Mef2) were investigated using QPCR and immunofluorescence. Exposing C2C12 cells to 20nM sodium arsenite delayed the differentiation process, as evidenced by a significant reduction in the number of multinucleated myotubes, a decrease in myogenin mRNA expression, and a decrease in the total number of nuclei expressing myogenin protein. The expression of mRNA involved in myotube formation, such as Ptgis and Mef2 mRNA, was also significantly reduced by 1.6-fold and 4-fold during differentiation. This was confirmed by immunofluorescence for Mef2, which showed a 2.6 fold reduction in nuclear translocation. Changes in methylation patterns in the promoter region of myogenin (-473 to +90) were examined by methylation-specific PCR and bisulfite genomic sequencing. Hypermethylated CpGs were found at -236bp and -126bp, whereas hypomeththylated CpGs were found at -207bp in arsenic exposed cells. Arsenic exposure

delays myotube differentiation and represses myogenin expression in part by altering DNA methylation in the myogenin promoter.

**Keywords:** arsenic; C2C12; myogenin; methylation; Mef2c; Ptgis; muscle; myocyte

## Introduction

Arsenic is present in drinking water systems around the world as both a natural element and as a contaminant due to normal geological processes and human disturbances (Mandal and Suzuki, 2002). Epidemiological studies indicate that long-term exposure to arsenic can result in adult-onset diseases, such as cancer, cardiovascular disease, diabetes, and skin lesions (Guha Mazumder, 2008; Mink et al., 2009; Platanias, 2009; Schuhmacher-Wolz et al., 2009; States et al., 2009). Although most studies have focused on outcomes such as cancer, epidemiological evidence indicates that arsenic can also act as a developmental toxicant (Cherry et al., 2008; Hopenhayn-Rich et al., 2000; Milton et al., 2005; Rahman et al., 2007; Rahman et al., 2009). Arsenic has been shown to cross the placental barrier in humans and rodents (Concha et al., 1998; Jin et al., 2010; Xie et al., 2007), and studies in rodent models have shown that dams exposed to arsenic in their drinking water had an increase in aborted fetuses, while their offspring had low birth weight and changes in locomotor activity (Ahmad et al., 2001; He et al., 2007; Jinet al., 2005; Waalkes et al., 2004; Waalkes et al., 2003).

Moreover, *in utero* arsenic exposure also alters muscle function and muscle-related protein expression in the offspring. For example, arsenic exposure *in utero* reduced the ability of smooth muscle to relax and resulted in changes in the vascular matrix by decreasing the amounts of collegen and elastin (Hays et al., 2008; Srivastava et al., 2007). In fish species, such as killifish, parental arsenic exposure increased tropomyosin and myosin light chain 2 expression in the offspring (Gonzalez et al., 2006). Zebrafish exposed to arsenic during embryogenesis showed altered heart development,

and their somite and neuromuscular patterning correlated to altered cell proliferation and genomic DNA methylation (Li et al., 2009). Alterations in muscular regulatory factors during zebrafish somitogenesis resulted in impaired development of fast skeletal muscle (Tilton and Tanguay, 2008). Additionally, arsenic exposure to frogs prior to metamorphosis resulted in reductions in swimming ability (Chen et al., 2009).

Skeletal muscle development occurs in several defined steps. Mesoderm-derived precursor cells become committed to the myogenic lineage and develop into myoblasts, which subsequently fuse to form myotubes. Later, myotubes mature into multinucleated highly specialized muscle fibers that show cross striation (reviewed in Brand-Saberi, 2005; Darabi et al., 2008). During muscle differentiation, myogenin is the key transcription factor that initiates the conversion of a skeletal muscle myoblast to a myotube (Buckingham et al., 2003). Recently, it has been reported that myogenin regulates several genes during muscle differentiation. For example, both *in vitro* and *in vivo* studies in mice indicate that conditional knockout of myogenin represses muscle stem cell differentiation along with reducing the expression of genes that are involved in myogenesis, including prostaglandin I2 synthesis (Ptgis), suppressor of cytokine signaling 3 (Socs3), and myocyte enhancer 2 (Mef2) (Meadows et al., 2008).

To date, it has been shown that methylation status on the myogenin promoter plays an important role in activation of myogenin. For example, in undifferentiated C2C12 myoblast cells, the myogenin promoter is maintained in a completely methylated status, which results in little myogenin expression. However, during differentiation, the promoter region undergoes demethylation within 48 hours and subsequently recruits its

transcription factors, such as MyoD, to initiate transcription (Fusoa et al., 2001; Lucarelli et al., 2001; Sartorelli and Caretti, 2005). Interestingly, when S-adenosylmethionine (SAM) is administered to C2C12 cells, it can repress expression of myogenin by delaying the demethylation of the 5'-flanking region on the myogenin gene (Fusoa et al., 2001; Lucarelli et al., 2001).

In many cells, arsenic undergoes enzymatic mono- and dimethylation, which is mediated by arsenic methyltransferase (As3MT) using SAM as a methyl group donor (Liu et al., 2007; Tsai et al., 2009; Vahter et al., 2007). However, SAM is also the universal methyl donor for the majority of methyltransferases that modify DNA, RNA, histones and other proteins, which dictates replication, transcription and translation fidelity, mismatch repair, chromatin modeling, epigenetic modifications and imprinting (Loenen, 2006). Since the SAM/methyltransferase pathway for biotransformation of arsenic overlaps with the DNA methylation pathway, one could hypothesize that the arsenic-induced adverse outcomes may be due to the imbalanced SAM pool within the organism. To this end, Zhao et al. have shown that arsenic induced global DNA hypomethylation occurred concurrently with aberrant gene expression and in the presence of reduced levels of SAM in mice (Zhao et al., 1997). Individuals chronically exposed to arsenic in their drinking water had hypermethylation on the promoters of p53 and p16 (Chanda et al., 2006), whereas whole genome hypermethylation in persons exposed to arsenic in drinking water has also been reported (Majumdar et al., 2009). Collectively, studies indicate that arsenic exposure results in both DNA hypomethylation and

hypermethylation, leading to aberrant gene expression (Benbrahim-Tallaa and Waalkes, 2008; Salnikow and Zhitkovich, 2008).

The objectives of the present study were to: 1) determine whether a low concentration of sodium arsenite delayed the differentiation of C2C12 cells from myoblasts to myotubes by repressing the transcription factor myogenin; 2) examine whether the reduction in myogenin would impact the expression of myogenic proteins involved in contraction and myotubes formation; and 3) determine whether DNA methylation patterns on the myogenin promoter were altered to better understand the molecular mechanism of arsenic.

**Methods**

*Arsenic exposure and quantification of multinucleated myocytes.*

The mouse myoblast C2C12 cell line (ATCC, Manassas, VA) was seeded at 860 cells/well in 6 well plates, and cultured with or without 20nm sodium arsenite in DMEM supplemented with 10% fetal bovine serum, 1% L-glutamine and 1% penicillin/streptomycin (growth media) at 37°C in a humidified incubator containing 5% $CO_2$. To differentiate the cells, the medium was replaced with DMEM supplemented with 2% horse serum, 1% L-glutamine and 1% penicillin/streptomycin (differentiation media) (Kubo, 1991) with or without 20nM arsenic.

Myotube formation was examined on differentiation days 0, 1, 2, 3, and 4 (n=6 per group per day by fixing the cells in methanol and incubating them in Giemsa stain. Cells were considered to be multinucleated if they contained 3 or more nuclei (Duan and

Gallagher, 2009). A multinucleation index was calculated by dividing the total number of cells by the number cells containing >3 nuclei.

*QPCR.*

C2C12 cells were plated at 5000 cells/100mm dish in either growth medium or in growth medium containing 20nM sodium arsenite, and on the fourth day, differentiation medium alone or differentiation medium containing 20nM sodium arsenite was added. Cells were harvested on days 0 or 4 (n=4 per group per day) and RNA was extracted using TRIreagent (Sigma-Aldrich, St. Louis, MO). RNA (2µg) was reverse transcribed into cDNA, and the expression of myogenin, myosin light chain 2, tropomyosin, prostaglandin I2 synthase (Ptgis), suppressor of cytokine signaling 3 (Socs3), and myocyte enhancer factor 2 (Mef2c) was quantified by QPCR using SybrGreen (SABiosciences, Frederick, MD) and the appropriate primers (Table 2. 1). Samples were run in triplicate and the entire experiment was repeated at least twice. The cycle threshold values (Ct) were converted into number of molecules per 100ng cDNA using known concentrations of the specific gene product, and then normalized using GAPDH.

*Cellular expression and localization of myogenin, tropomyosin, and Mef2c.*

C2C12 cells were seeded in Lab-tek II eight well coverglass slides (Nunc) at a concentration of 100 cells/well. Cells were cultured with or without 20nM sodium arsenite as described above and examined for specific protein expression on differentiation days 0, 1, 2, 3, and 4 (n=8 chambers per protein per group per day). Cells were blocked in 1% bovine serum albumin, 0.1% Triton-X100 in PBS, and incubated with the appropriate primary antibody for 1 hour (myogenin: 1:100 dilution, Imgenex;

tropomyosin: 1:50 dilution, Santa Cruz Biotechnologies; Mef2: 1:50 dilution, Santa Cruz). The secondary antibody (1 □g/mL) conjugated to Alexa Flour 488 (Invitrogen, Carlsbad, CA) was incubated with the cells, which were counterstained with DAPI (Invitrogen. Cells were examined by conventional immunofluorescence on a Ti Eclipse Inverted Microscope (Nikon, Melville, NY). Myogenin and Mef2c expression was quantified by determining the percentage of nuclei expressing each protein. Tropomyosin expression was quantified by determining the mean pixel intensities in individual cells by examining 4 fields per slide.

*Bisulfite genomic sequencing of the myogenin promoter.*

Bisulfite genomic sequencing PCR was performed using specific primer sets to amplify nucleotides −473 to +90 relative to the transcription start site (TSS) of myogenin (Table 2.1). C2C12 cells were seeded at $1.5x10^5$/150mm tissue culture plate and were cultured with or without 20nM sodium arsenite as described above. Genomic DNA was extracted on differentiation day 2 using Qiagen's Genomic DNA extraction kit (Valenic, CA; n=3 plates per group). Sodium bisulfite treatment of the DNA was performed using the EpiTect Kit (Qiagen). The BS-PCR reaction was performed for 40 cycles and the products were cloned into the pCR2.1 vector (Invitrogen) and transformed into Top10 chemically competent *E. coli* (Invitrogen). Twenty to thirty randomly selected clones from the control and arsenic-exposed group were sequenced with the M13 reverse primer to determine whether each one of the 12 CpG sites was methylated or not. A chi-square test was used to determine statistical significance between control and arsenic-exposed cells.

## Results

### *Arsenic delays C2C12 muscle cell differentiation*

Initial cell viability assays demonstrated that the $LC_{50}$ of sodium arsenite in C2C12 cells was 5μM (data not shown), so we used a 250-fold lower concentration to determine whether arsenic caused a delay in C2C12 cell differentiation. Cells were cultured either with or without 20nM sodium arsenite in growth media for 3 days and then in differentiation medium for up to 4 days. Both control and arsenic-exposed cells appeared predominantly as myoblasts during culture in growth medium (Fig. 2.1A-B). After the switch to differentiation medium, myotube formation was reduced in the arsenic exposed cells compared to the controls (Fig. 2.1C-D). Quantifying the number of multinucleated cells as an indicator of myotube formation indicated that the number of multinucleated cells was significantly reduced between 1.5- and 2.9-fold in the cells exposed to arsenic (Fig. 2.1E).

### *Myogenin transcript and nuclear localization is reduced during arsenic exposure*

Since arsenic appeared to delay C2C12 cell differentiation and multinucleation, the expression of myogenin, the transcription factor that initiates the differentiation of a myoblast to a myotubes, was examined. When C2C12 cells were cultured in growth medium, there was very low expression of myogenin mRNA in both control and arsenic-exposed cells. However, by day 2 of differentiation, myogenin transcript was significantly reduced 7-fold in the arsenic-exposed cells, and on day 4, myogenin RNA levels were still reduced by 4.1-fold in the arsenic-exposed cells compared to control cells (Fig. 2.2 H). The reduced levels of RNA also correspond with alterations in

myogenin nuclear translocation. In growth medium, myogenin is localized in the cytoplasm in both control and arsenic-treated cells (Fig. 2.2 A and D). After 2 days of differentiation, myogenin in the control cells shifts to the nuclei (Fig. 2.2 B and E), as the number of nuclei expressing myogenin was significantly reduced in the arsenic-exposed C2C12 cells by 1.6-fold (Fig 2.2 G). The reduced expression of myogenin is likely causing the delay in differentiation in arsenic-exposed cells.

***Altered Cellular Expression and Localization of Muscle Proteins***

Since myogenin expression was reduced after arsenic exposure, we next determined whether this would lead to alterations in myogenic proteins. The expression of tropomyosin mRNA, a part of the contractile apparatus, was unchanged after arsenic exposure (Fig. 2.3 A). Its protein expression, as examined by immunofluorescence, was also not altered (data not shown). Similarly, Socs3, a protein that has been reported to induce the differentiation of myoblasts (Spangenburg, 2005), was also not changed after exposure to arsenic (Fig. 2.3 C). However, the RNA expression of Mef2c, a transcription factor that is expressed during late myogenesis (McDermott et al., 1993) that appears to control myofiber subtype (Potthoff et al., 2007b; Wu et al., 2000), was reduced by 4-fold after arsenic exposure (Fig. 3B). Additionally, Ptgis, which is thought to be involved in myoblast fusion (Bondesen et al., 2007; Prisk and Huard, 2003), was also significantly reduced by 1.6-fold in cells treated with 20nM arsenic (Fig. 2.3 D). Immunofluorescence corroborated the QPCR results for Mef2c, showing a reduction in nuclear translocation by 2 to 2.7-fold in the arsenic treated cells (Fig. 2.4 G).

### *Changes in Myogenin Promoter Methylation*

To investigate one of the potential mechanisms responsible for the changes in myogenin expression, we examined methylation patterns on the myogenin promoter. Bisulfite sequencing PCR was performed to amplify the nucleotides -473 to + 90 relative to the transcription start site (TSS) of myogenin, which contains a total of 12CpG sites (Fig. 2.5A). Chi-square analysis indicated that hypermethylated CpGs were found at CpG No. 3 and 8 (-236 and -126, respectively), whereas hypomethylated CpGs were found at CpG No. 7 (-207) in arsenic exposed cells (Fig. 2.5 B and C). This increase in methylated CpG sites on the myogenin promoter may be, in part, responsible for the reduction in myogenin mRNA and nuclear localization in arsenic-exposed C2C12 cells.

## Discussion

These results illustrate that 20nM sodium arsenite can alter myoblast differentiation by reducing the expression of the transcription factors myogenin and Mef2c, which is likely due to changes in promoter methylation patterns. To our knowledge, this is the first study that has examined the affects of low nanomolar arsenic concentrations and its effect on the development of skeletal muscle cells. Previous studies have used much higher arsenic concentrations, ranging from 0.1 – 267μM, when examining cardiac, skeletal, and smooth muscle development *in vivo* or *in vitro* (He et al., 2007; Lantz et al., 2008; Li et al., 2009; Yen et al., 2010). For example, when zebrafish embryos were exposed to 2mM arsenic, the embryos had malformations of the musculature like dorsal curvature and improper heart development (Li et al., 2009). *In utero* and postnatal arsenic exposure of 100ppb in mice increased the amount of smooth

muscle mass and actin in the lung (Lantz et al., 2008). Additionally, when pregnant mice ingested drinking water containing 20ppm or higher sodium arsenite, there was a decrease in their fecundity, and if offspring were produced, their placenta experienced defective formation of blood vessels (He et al., 2007). All these studies provide evidence that arsenic can act as a developmental toxicant.

During the development of skeletal muscle, myogenin is the transcription factor that regulates the differentiation of a myoblast to a myotubes. Myogenin knockout mice die shortly after birth due to malformation of skeletal muscles and lack of diaphragm formation (Hasty et al., 1993). In the present study, myogenin mRNA was reduced in the arsenic-treated cells during days 2, 3 and 4 of differentiation A recently published study also saw reductions in myogenin after exposure of C2C12 cells to arsenic (Yen et al., 2010), although the arsenic levels used in that study were an order of magnitude higher than in our study. In our research, the reduction in myogenin coincided with a delay in differentiation and a reduction in the total number of multinucleated myotubes, which indicates that nanomolar arsenic levels are having a physiological effect on cellular differentiation.

Additionally, our results indicate that Mef2c gene expression and nuclear translocation was also reduced an average of 2.6-fold in arsenic exposed cells. Mef2c is a transcription factor that is essential for muscle differentiation and is activated by myogenin (Dodou and Xu, 2003). The promoter for myogenin contains a Mef2 binding site, which provides a way for maintaining myogenin expression in muscle cells (Edmondson et al., 1992; Molkentin and Olson, 1996). Thus, the 2 sets of transcription

factors work cooperatively to regulate skeletal muscle genes Mef2c deletion in the skeletal muscle of mice results in lethality before birth (Lin et al., 1997) and disorganized myofibers, which are due to disorganized sarcomeres and the loss of M-line encoding proteins such as myomesin (Potthoff et al., 2007a). Like myogenin, Mef2 appears to play a role in myotube and myofiber formation. To our knowledge, this is the first time that arsenic has been shown to alter Mef2 levels. However, whether the reduction in Mef2c is an indirect effect due to the loss of myogenin or whether arsenic can directly alter myogenin expression is unclear.

It has been shown that the methylation status in the myogenin promoter correlates with its expression and with muscle differentiation (Lucarelli et al., 2001; Palacios et al., 2010). To this end, we examined the DNA methylation patterns in the myogenin promoter region. Although there is a weak CpG island in myogenin promoter, there is a relatively high density of CpGs from -473 bp to +90 bp,. The results indicate that there are two hypermethylated CpGs at -236 and -126 in the arsenic exposed C2C12 cells. Interestingly, a hypomethylated CpG at -207 was also detected in the arsenic treatments. Since the methylated CpGs may directly interfere gene activation by preventing the binding of potential transcription factors or modifications of histone tails, identification of potential arsenic mediated transcription factors that could interact with the hypomethylated CpG at -207, as well as hypermethylated CpG at -236 and -126, is currently under study. Additionally, chromatin immunoprecipitation (ChIP) were also performed to investigate the relationship between DNA methylation and histone modification at H3K9-Me2/Me3 on the myogenin promoter from -481bp to -28bp. Our

results show no correlation between the differentially methylated CpGs and the formation of transcriptional repressive heterochromatin in myogenin promoter, indicating that H3K9 methylation does not play a major regulatory role in myogenin expression in arsenic treatment (data not shown). Moreover, we did examine the expression of DNMT1, DNMT3a, and DNMT3b, as well as cellular concentrations of SAM, but there were no changes between control and arsenic-exposed cells (data not shown). Recently, demethylation of CpGs in the myogenin promoter from -192bp to +58bp has been reported in during differentiation of C2C12 cells (Palacios et al., 2010). Our results show a hypermethylated CpG (CpG #8, -126bp) in arsenic-exposed C2C12 cells. Moreover, an enhancer binding site (-255bp to -241bp) was predicted in the myogenin promoter, which is in the same area where we identified a hypermethylated CpG (CpG #3, -236bp). Therefore, our findings suggest that these two hypermethylated CpGs may reduce the transcriptional activation of myogenin, or block enhancer binding in the myogenin promoter during arsenic exposure.

Collectively, our results indicated that 20nM sodium arsenite results in delayed differentiation in C2C12 mouse myocyte cells. The delayed muscle differentiation was caused by the reductions in myogenin mRNA expression and reduced nuclear localization of myogenin protein. Repressed myogenin expression was likely due to abnormal DNA methylation in the myogenin promoter, as well as the reduced expression of Mef2c, which is a transcription factor that contributes to myogenin expression. A delay in muscle cell differentiation could lead to improper organization of myofibers,

which ultimately can have serious physiological consequences on the organism at arsenic concentrations that are below the current drinking water standard.

**Acknowledgments**

This work was supported by NIH grants ES016640 and ES016640-01S1, and CSREES/USDA under project #SC-1700380. It is technical contribution No. 5835 of the Clemson University Experiment Station. The authors would also like to thank Terri Bruce from the Clemson University Imaging Facility for technical assistance.

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**Table 2.1 Primers for QPCR and bisulfite sequencing**

*QPCR*

| Primer | Forward (5' to 3') | Reverse (5' to 3') | Annealing temp ($^{o}$C) |
|---|---|---|---|
| Gapdh | tgcgacttcaacagcaactc | atgtaggccatgaggtccac | 62.5 |
| Myogenin | ccaacccaggagatcatttg | acgatggacgtaagggagtg | 61 |
| Ptgis | tgccagcttccttaccaggatgaa | taagagtgtgggtccaggagaaca | 61 |
| Tropomyosin | tgaaagtcattgaaagccgagccc | agcaatgtgcttggcctctttcag | 56 |
| Mef2c | aggatcaccggaacgaattccact | gcatgcgcttgactgaaggactt | 61 |
| Socs3 | agcagatggagggttctgctttgt | attggctgtgtttggctccttgtg | 61 |

*Bisulfite Sequencing*

| Primer | Forward (5' to 3') | Reverse (5' to 3') | CpG # |
|---|---|---|---|
| BS-MSP-1 | ttttggattatggaggagaga | ccccaccctacaaacctac | #1 to #7 |
| BS-MSP-2 | ggaaggggaattatatgtaattt | aaataattttccccatcataaa | #8 to #11 |

BS-MSP: Bisulfite sequencing- methylation specific PCR

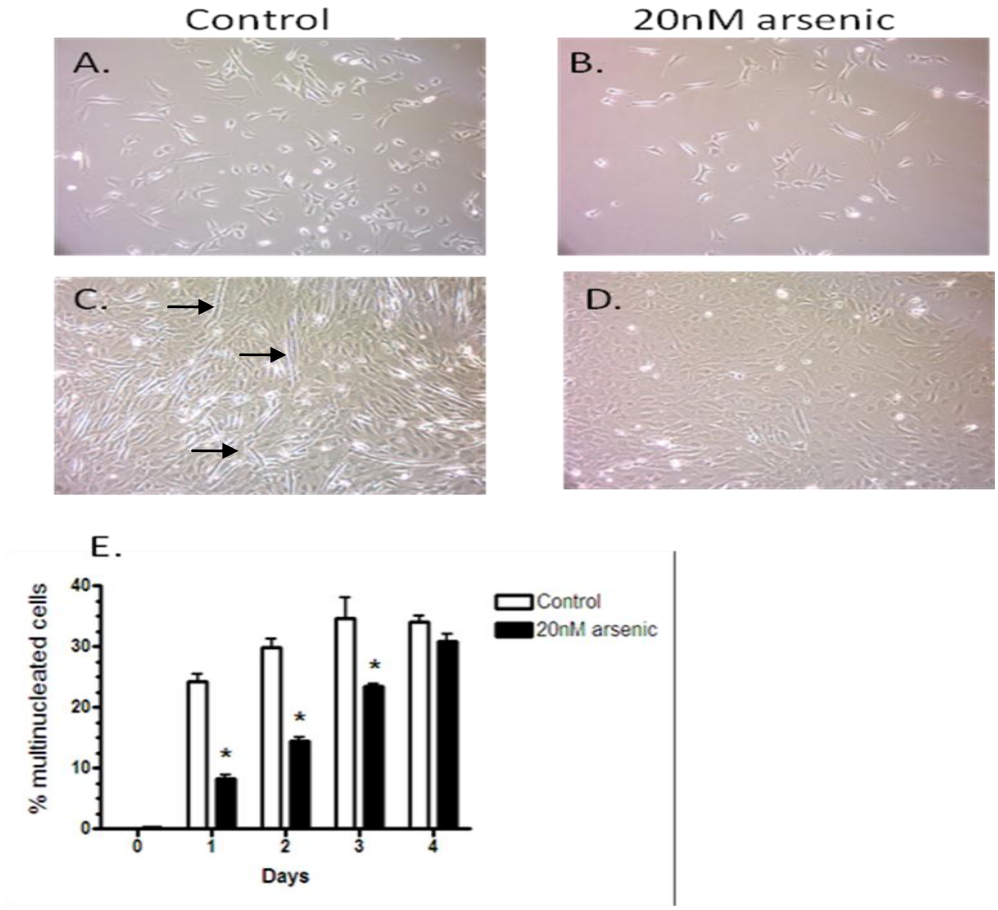


**Fig. 2.1 Arsenic exposure delays C2C12 cell differentiation.** After 3 days of growth, myoblast cells grown in growth medium with or without 20nM arsenic (A and B) were switched to differentiation medium for 4 days (C and D). Myotube formation is indicated by the arrows. Photographs are representative examples from 4 plates/day/group. Myotubes that had 3 or more nuclei were counted from 4 random areas per plate (n=4 plates/day/group) (E) and statistical differences (*) in the percentage of myotubes formed was determined by Student's t-test ($p < 0.05$).

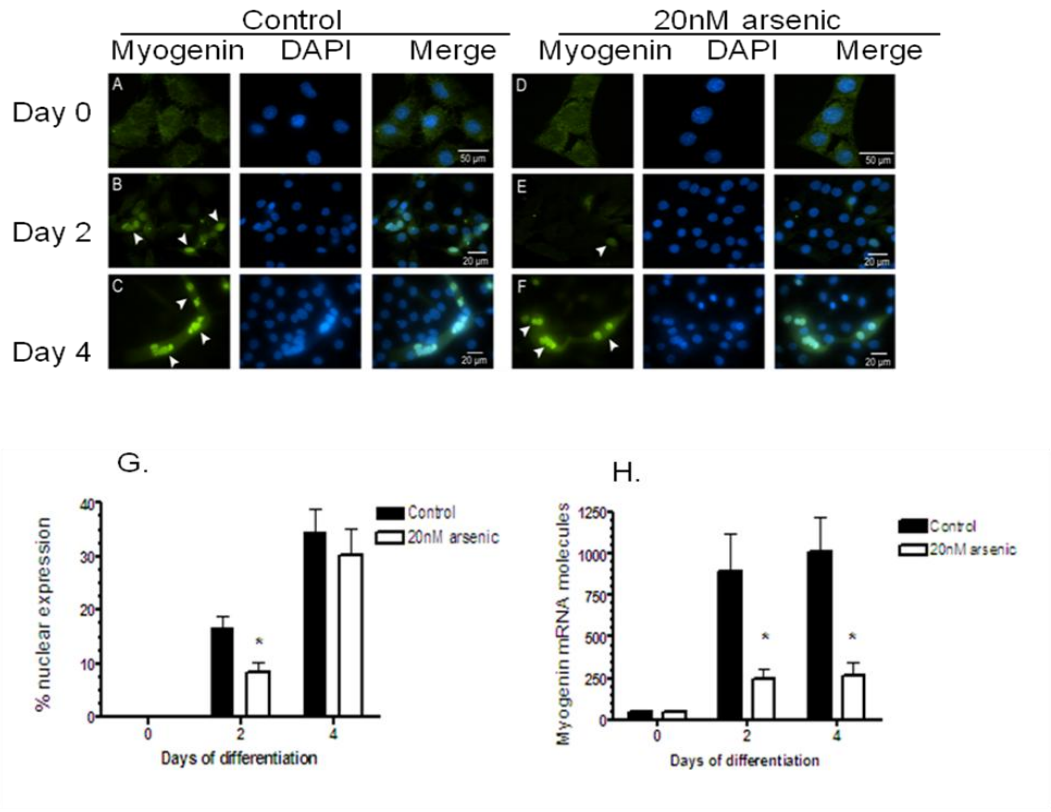


**Fig. 2.2 Arsenic alters myogenin nuclear localization and mRNA expression**. Control cells on day 0, 2, and 4 of differentiation (A-C) and 20nM arsenic treated cells on day 0, 2, and 4 of differentiation (D-F) were used to examine myogenin localization by immunofluorescence. White arrows indicate nuclei that are expressing myogenin. Pictures are representative examples from 4 wells/day/group. The percentage of nuclei expressing myogenin (G) was determined by counting the number of nuclei expressing myogenin per total cells in each photo taken ($n \geq 5$). Myogenin RNA expression was determined by QPCR (H). Values were normalized against GAPDH as a housekeeping gene, with each sample run in triplicate (n=4 plates/day/group). Statistical differences (*) were determined by Student's t-test ($p < 0.05$).

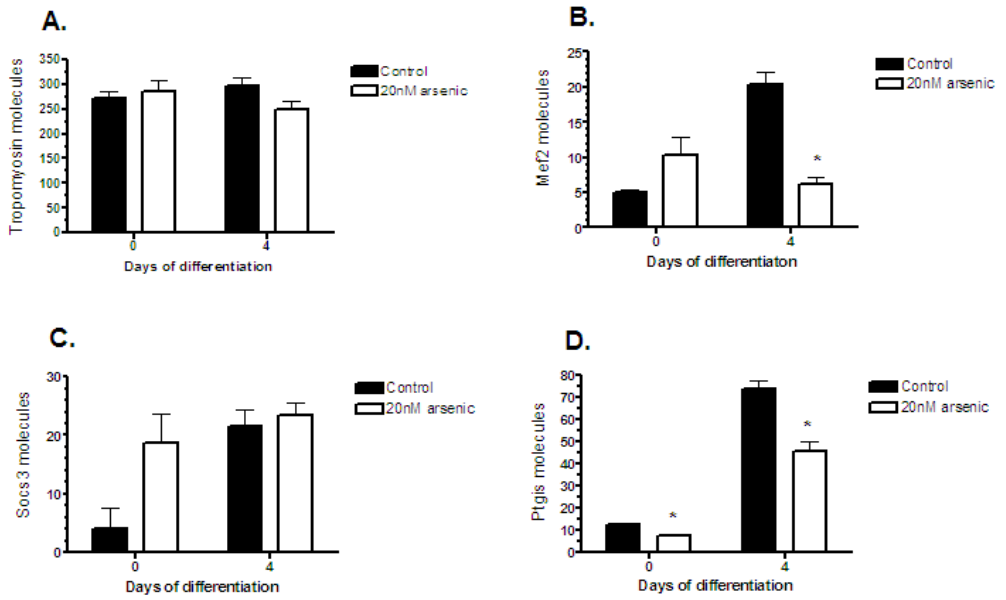


**Fig. 2.3 Changes in the mRNA expression of tropomyosin, Mef2C, Soc3, and Ptgis.** Tropomyosin (A), Mef2C (B), Socs3 (C), and Ptgis (D) RNA expression was determined by QPCR. The data is expressed in number of molecules/100ng cDNA $\pm$ standard deviation. Values were normalized against GAPDH as a housekeeping gene, with each sample run in triplicate (n=3-5 plates/day/group). Statistical differences (*) were determined by Student's t-test ($p < 0.05$).

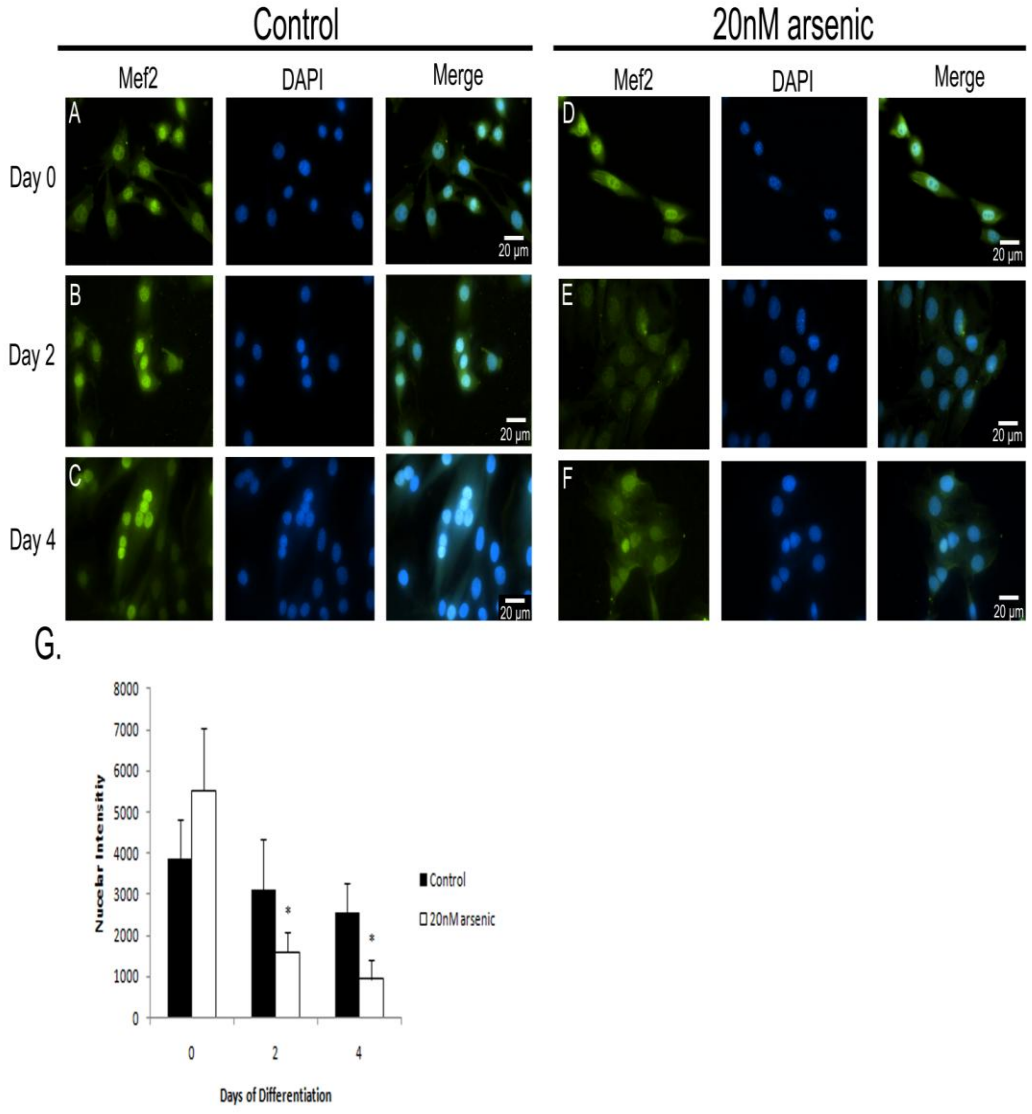


**Fig. 2.4 Immunofluorescence of Mef2C.** Mef2C protein expression was examined on day 0, 2, and 4 in control (A-C) and 20nM arsenic treated cells (D-F). Pictures are representative examples from 4 wells/time point/group. Differences in expression between control and treated cells (G) were determined by measuring the intensities of the nuclei in each photo taken ($n \geq 5$). Nuclear intensities were normalized to background values and statistical differences (*) were determined by Student's t-test ($p < 0.05$).

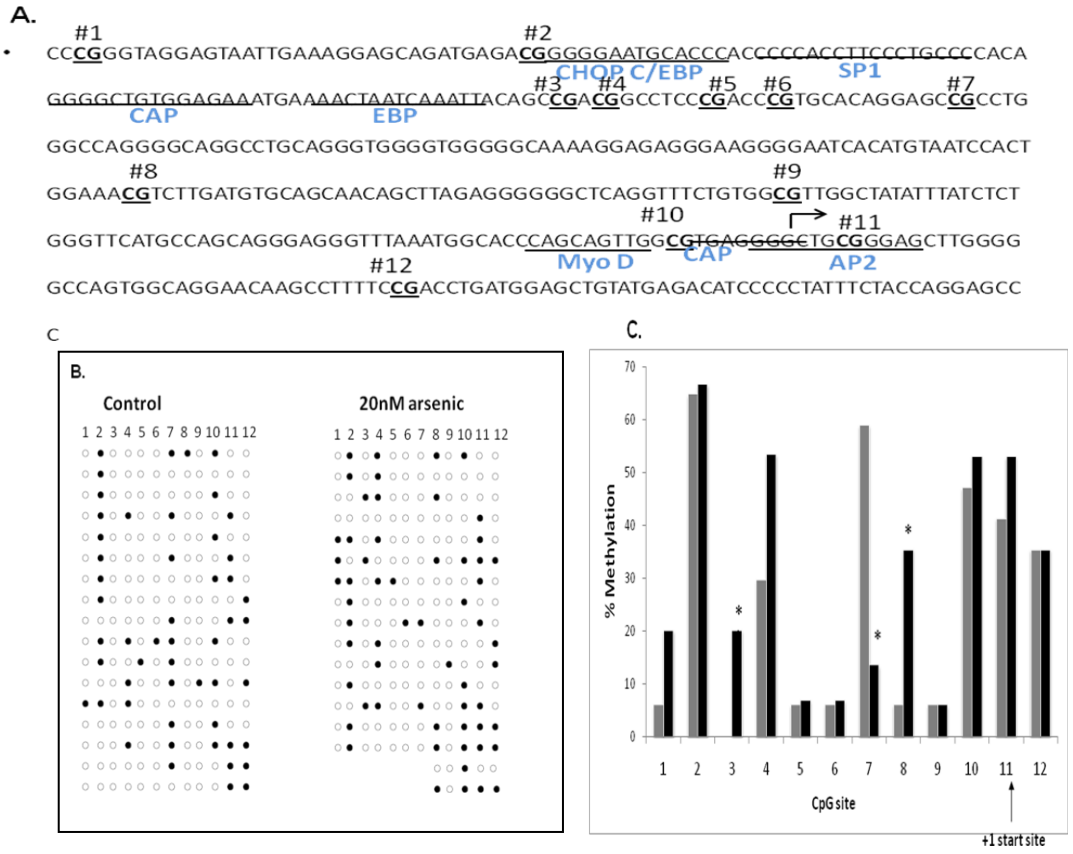


**Fig. 2.5 Arsenic alters the methylation pattern on the myogenin promoter.**

A portion of the myogenin promoter (-473 to +90) was examined for CpG sites and potential transcription factor binding sites by MOTIF Search (http://motif.genome.jp/). Bolded nucleotides indicate a CpG site and the number denotes its order. Underlines signify putative transcription factor binding sites, and the transcriptional start site is indicated with an arrow (A). Bisulfite sequencing was performed to determine the methylation status at each of the CpG sites in clones from control and 20nM arsenic exposed cells. Open circles denote nonmethylated sites and closed circles denote methylated sites (B). The percentage of methylated Cs was calculated for each CpG site (C), and statistical differences (*) were determined using a Chi-Square test ($p < 0.05$).

# CHAPTER THREE

# SODIUM ARSENITE REPRESSES THE EXPRESSION OF MYOGENIN IN C2C12 MOUSE MYOBLAST CELLS THROUGH HISTONE MODIFICATIONS AND ALTERED EXPRESSION OF EZH2 AND IGF-1

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This manuscript has been submitted to *Toxicology* and is in the format required of that journal.

**Abstract**

Arsenic is toxicant commonly found in water systems around the world. Chronic arsenic exposure can result in adverse developmental effects such as increased neonatal death, stillbirths, and miscarriages, as well as low birth weight and altered locomotor activity. Our previous study indicated that 20nM sodium arsenite exposure to C2C12 mouse myocyte cells resulted in delayed myoblast differentiation, because of reductions in myogenin expression, the transcription factor that differentiates myoblasts into myotubes. In this study, several mechanisms by which arsenic could alter myogenin expression were examined. Arsenic exposure to differentiating C2C12 cells resulted in altered histone methylation and acetylation status on the myogenin promoter, as 20nM arsenic increased H3K9 dimethylation (H3K9me2) and H3K9 trimethylation (H3K9me3) by 3-fold near the transcription start site of myogenin, which is indicative of increased heterochromatin formation, and arsenic exposure reduced H3K9 acetylation (H3K9Ac) by 0.5-fold, which is indicative of reduced euchromatin formation. In addition to the altered histone remodeling status on the myogenin promoter, protein and mRNA levels of Igf-1, a myogenic growth factor, were significantly repressed by arsenic exposure. Moreover, a 2-fold induction of Ezh2 expression, as well as an increased recruitment of Ezh2 (3.3-fold) and Dnmt3a (~2-fold) to the myogenin promoter at the transcription start site (-40 to +42), were detected in the arsenic-treated cells. Together, we conclude that the repressed myogenin expression in arsenic-exposed C2C12 cells was likely due to a combination of the reduced expression of Igf-1, the enhanced nuclear expression and

promoter recruitment of Ezh2, and the altered histone remodeling status on myogenin promoter (-40 to +42).

**Keywords**: Arsenite; myocyte; Myogenin; Histone remodeling; Igf-1; Ezh2

## Introduction

Arsenic is a toxicant commonly found in water systems around the world. Chronic arsenic poisoning is a global health problem affecting millions of people (Cherry 2008; McDonald 2007; Medrano et al. 2010; Wang et al. 2009) which can result in cancer, central nervous system and sensory deficits, effects on development, and neuromuscular deficits (Andrew et al. 2007; Benbrahim-Tallaa and Waalkes 2007; Kozul et al. 2009; Mohammad et al. 2009). Unfortunately, the mechanisms responsible these multiple adverse outcomes remain largely unclear and likely are multi-factorial.

Arsenic is also a developmental toxicant. In humans and rodents, arsenic can traverse the placenta and this exposure results in adverse developmental effects, such as increased neonatal death and stillbirths (Agusa et al. 2010; Concha et al. 1998; Markowski et al. 2011; Raqib et al. 2009; von Ehrenstein et al. 2006). In fish, arsenite-exposed zebrafish embryos have reduced survival and delayed hatching, malformations in the spinal cord and heart, and disordered motor axon projections (Li et al. 2009). Recently, negative effects of arsenic on early embryonic development have been reported (Flora and Mehta 2009; Stummann et al. 2008). Results from mouse embryonic stem cells indicate that 10 mM arsenic inhibits cardiac differentiation by reducing the formation of embryoid bodies, which is an essential step for stem cell differentiation (Stummann et al. 2008). Arsenic (1ppb) treated human stem cells show altered pluripotency and significant down regulation of genes indicative of all the three germ layers (Flora and Mehta 2009).

Arsenic-mediated adverse effects on muscle differentiation have also been reported. In killifish (*Fundulus heteroclitus*), arsenic exposed parents had offspring with increased trunk curvatures, which was correlated with changes in myosin light chain, type II keratin, tropomyosin, and parvalbumin expression in the hatchlings (Gonzalez et al. 2006). Arsenic exposure to mouse C2C12 myoblasts delayed their differentiation into myotubes, likely due to a reduction in the expression of myogenin (Steffens et al. 2011). In rodent models, arsenic suppresses the regeneration of injured muscles (Yen et al. 2010), alters pulmonary structure and function *in utero* by increasing the smooth muscle actin in the lung (Lantz et al. 2009), and disrupts the smooth muscle integrity around the blood vessels in the heart (Hays et al. 2008). Collectively, these results suggest that arsenic acts as a developmental toxicant by affecting the development of the musculature.

The development of skeletal muscle is regulated by several myogenic transcription factors, such as Myo D, myogenin, and myocyte enhancer factor 2 (Mef2). In muscle differentiation, MyoD and Mef2 are early markers, which are expressed during myoblast determination, and they then regulate myogenin, which induces terminal differentiation by converting myoblasts into myotubes (Carvajal and Rigby 2010; Gianakopoulos et al. 2011; Yokoyama and Asahara 2011). Moreover, other signaling molecules, such as insulin-like growth factor 1 (Igf-1) and myostatin, regulate myogenin expression *via* the PI3K/AKT pathway during skeletal muscle differentiation (Alzhanov et al. 2010; Artaza et al. 2002; Yang et al. 2007). In addition, chromatin-modifying enzymes also regulate muscle development by epigenetically repressing myogenic

transcription factors (Albert and Peters 2009; McDonald and Owens 2007; Ohkawa et al. 2007).

Recently, arsenic-induced alterations in DNA methylation and histone modifications have been suggested to play a role in carcinogenesis and the fetal origins of diseases (Arita and Costa 2009; Baccarelli and Bpllati 2009; Ren et al. 2010). Altered DNA methylation may occur since the pathway for biotransformation of arsenic also relies on methylation (Baccarelli and Bpllati 2009; Ren et al. 2010; Vahter 2009). To this end, studies have shown that arsenic exposure results in both hypermethylation and hypomethylation at global and gene specific levels, thereby leading to aberrant gene expression. For example, mice exposed to arsenic have reduced p16 expression in lung tumors due to hypermethylation of the p16 gene. In humans, arsenic induces DNA hypermethylation in the promoters of the p53 and p16 genes (Benbrahim-Tallaa and Waalkes 2007; Chanda 2006; Salnikow and Zhitkovich 2008; Zhou et al. 2008). Moreover, a significant relationship between arsenic exposure and promoter hypermethylation of two tumor suppressor genes, PRSS3 and RASSF1A, was identified in a population-based study of human bladder cancer (Marsit et al. 2006). In addition to DNA methylation, arsenic also has a role in histone modification. H3K9 dimethylation (H3K9me2) and H3K9 trimethylation (H3K9me3), both markers of gene silencing, were induced at the global level in human lung carcinoma A549 cells and in normal human bronchial epithelial BEAS-2B cells upon exposure to 2.5μM arsenic (Zhou et al. 2008). Acetylation of K9 in histone H3 (H3K9 Ac), which represents transcriptional activation, was reduced by 50% at a global level in human UROtsa cells upon exposed to arsenic

(3μM) for 7 days (Chu et al. 2011). Moreover, arsenic represses steroid hormone-mediated transcription by disrupting acetylation of K18 in histone H3 (H3K18) at the estrogen-responsive pS2 promoter (Barr et al. 2009). Collectively, these and other reports suggest that arsenic can epigenetically alter gene expression *via* either DNA methylation or histone modifications.

Our previous studies indicate that 20nM sodium arsenite delays the differentiation of C2C12 mouse myoblast cells by repressing myogenin expression, which was likely due to the altered DNA methylation patterns on myogenin promoter and the decreased nuclear translocation of Mef2 (Steffens et al. 2011). Since the potential regulatory mechanisms responsible for the arsenic-induced delay in muscle differentiation remain largely unclear, the objectives of the present study were to examine whether the arsenic-induced abnormal methylation patterns would lead to changes in chromatin structures on myogenin promoter and investigate whether other muscle transcription and growth factors were altered by arsenic exposure.

**Methods**

*Cell culture.*

C2C12 myoblasts were maintained in growth medium (GM) consisting of Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, 1% L-glutamine and 1% penicillin/streptomycin solution. Differentiation medium (DM) was DMEM containing 2% horse serum, 1% L-glutamine and 1% penicillin/streptomycin solution (Kubo 1991). For differentiation studies, $15x10^4$ cells were seeded in a 150mm dish with or without 20nM arsenic as sodium arsenite, and then cultured for in growth

medium for 3 days (GM3). On day 4, the culture medium was changed to differentiation medium (DM) with or without 20nM arsenic to induce myotube differentiation. Cells were cultured for 2 days (DM2) and then harvested.

*Igf-1 mRNA Expression.*

C2C12 cells were cultured with or without 20nM arsenic as sodium arsenite as described above, harvested at differentiation hour 12, 24, 36, and 48 (n=3 per group per time point), and total RNA extracted. The mRNA expression of Igf-1 was quantified using $RT^2$ SYBR Green Supermix (Qiagen) according to manufacturer's instructions. The oligonucleotides used for qPCR were Igf-1 Forward: 5'-GAC CGA GGG GCT TTT ACT TCA-3', Reverse: 5'-GGA CGG GGA CTT CTG AGT CTT-3'; and Gapdh Forward: 5'-TGC GAC TTC AAC AGC AAC TC-3', Reverse: 5'-ATG TAG GCC ATG AGG TCC AC-3'. Samples were run in triplicate, and relative gene expression was calculated using the comparative threshold (Ct) method (Livak and Schmittgen 2001).

*Chromatin Immunoprecipitation (ChIP).*

C2C12 cells were cultured with or without 20nM sodium arsenite as described above and harvested on differentiation day 2 (DM2) (n=4 per group per day)**.** Chromatin preparation and immunoprecipitation was performed according to standard protocols (Abcam Inc., Cambridge, MA). The antibodies used for ChIP assays were: anti-Ezh2 (Millipore Inc., Temecula, CA), anti-Mef2 (Santa Cruz Biotech Inc., Santa Cruz, CA), anti-Dnmt3a and –Dnmt3b (Imgenex Inc., San Diego, CA), and anti-H3K9 Ac, -H3K9 Me2, and -H3K9 Me3 (Abcam). Normal rabbit and mouse IgG (Santa Cruz) were used as

negative controls. Quantification of precipitated DNA was performed by qPCR using SYBR green and myogenin promoter-specific primers. The oligonucleotides used for ChIP assays were ChIP 1 Forward: 5'-TAA TCA AAT TAC AGC CGA CGG CCT CC-3', Reverse: 5'-GCT GCA CAT CAA GAC GTT TCC AGT-3'; ChIP 2 Forward: 5'-CGT CTT GAT GTG CAG CAA CAG CTT-3', Reverse: 5'-CAT TTA AAC CCT CCC TGC TGG CAT-3'; and ChIP 3 Forward: 5'-GGG TTT AAA TGG CAC CCA GCA GTT-3', Reverse: 5'-TCA TAC AGC TCC ATC AGG TCG GAA-3' (see Fig. 2 for specific locations of each ChIP assay). Relative enrichment of the myogenin promoter DNA relative to a no-antibody control was calculated based on difference in threshold (Ct) values ($2[Ct_{antibody} - Ct_{IgG}]$)(Nelson et al. 2006).

*Immunofluorescence analysis of Ezh2, MyoD, and Igf-1.*

C2C12 cells were seeded in Lab-tek II 8-well chamber slides (Nunc) at a concentration of 100 cells/well. To determine the expression Ezh2 and MyoD, cells were cultured with or without 20nM sodium arsenite as described above and examined for specific protein expression on differentiation days 0 (GM3) and 2 (DM2) (n=4 wells per protein per group per day). For Igf-1 expression, cells were examined on differentiation day 0 (GM3) and differentiation hour 24, 36, and 48 (DM2). Cells were fixed with either 4% paraformaldehyde at room temperature for 20 minutes or methanol at -20 ° C for 5 minutes, blocked in 1% bovine serum albumin, 0.1% Triton-X100 in PBS, and incubated with the appropriate primary antibody for 1 hour at a 1:100 dilution. The secondary antibody (1μg/mL) conjugated to Alexa Flour 488 (Invitrogen, Carlsbad, CA) was incubated with the cells, which were counterstained with DAPI (Invitrogen). Cells were

examined by conventional immunofluorescence on a Ti Eclipse Inverted Microscope (Nikon, Melville, NY). Ezh2 and MyoD expression was quantified by determining the intensities of nuclear staining in six randomly selected fields per well, for a total of four independent wells/time point/group. Intensities were expressed as fold-change relative to control groups. For Igf-1 quantification, the number of nuclei expressing Igf-1 was counted from six randomly selected fields per chamber, for a total of four independent wells/time point/group.

*Statistical analysis.*

Student's *t*-test was used for all statistical analysis and *p*-values of $<0.05$ were considered to be statistically different.

**Results**

***Arsenic exposure reduces Igf-1 expression in C2C12 cells.***

Igf-1 is a potent inducer of muscle differentiation (Musaro et al. 1999). Although the Igf-1 responsive elements in the myogenin promoter remain unclear during muscle differentiation, it has been shown that Igf-1 induces myogenin expression by targeting Mef2 and MyoD to the myogenin promoter in C2C12 cells through the Igf-1/PI3K/AKT pathway (Xu and Wu 2000). To this end, we examined whether arsenic exposure would alter the expression of Igf-1 in C2C12 cells. I*mmunofluorescence* staining showed no significant alternation of Igf-1 expression in myoblasts (GM3) with or without arsenic exposure (Fig. 1A). A time course study was conducted to examine Igf1 expression at DM1 (24 hours), DM1.5 (36 hours), and DM2 (48 hours). Interestingly, increased Igf-1 protein in the nuclei was observed in differentiating myoblasts, compared with

undifferentiated myoblasts (see the arrows in Fig. 1A) at DM1 and DM1.5, and by DM2, a significant reduction in cellular Igf-1 was observed from cells differentiated in 20nM arsenic (Fig. 1A). An 11.8-fold and 5-fold reduction in the number of nuclei expressing Igf-1 was observed in the arsenic-exposed C2C12 cells at DM1 and DM1.5 (Figure 1B). qPCR corroborated the immunofluorescence, showing a significant reduction in Igf-1 mRNA expression by 2.3-fold, 1.8-fold, and 2.1-fold in the arsenic-treated cells at differentiation hour 24, 36, and 48, respectively (Fig. 1C).

***Arsenic exposure alters histone remodeling status on the myogenin promoter near the transcription start site in C2C12 cells.***

Our previous study indicated that 20nM sodium arsenite exposure to C2C12 mouse myocyte cells resulted in delayed differentiation because of a reduction in myogenin expression. We hypothesized that the repressed myogenin expression was due to abnormal DNA methylation patterns on the myogenin promoter (Steffens et al. 2011). Since DNA methylation patterns may affect gene expression by causing histone remodeling (Bird 2002) or altering transcription factor availability (Palacios et al. 2010), ChIP assays were performed to examine histone remodeling status on the myogenin promoter after arsenic exposure to C2C12 cells. Markers of heterochromain (H3K9 Me2 and H3K9 Me3) and euchromatin (H3K9 Ac) were examined by looking at three different areas on the myogenin promoter. ChIP 1 encompasses -114 to –251 of the myogenin gene, and arsenic exposure hyper-and hypo-methylates CpG#2 at -236 and CpG#5 at -207, respectively. ChIP 2 encompasses -31 to -128, which contains a hypermethylated CpG#6 at -126 and Mef2 binding site. ChIP 3 encompassess -40 to +42,

and contains an E-box at -13 to -18 and the transcription start site (Fig. 2A). Results from ChIP 1 and ChIP 2 showed no significant differences between control and arsenic groups with the three histone markers (Fig. 2B). However, chromatin precipitated from ChIP 3 (-40 to +42 of the myogenin promoter) indicated that H3K9 Me2 and –M3 were significantly induced by 3-fold (Fig. 2B), which is indicative of increased heterochromatin formation, while H3K9 Ac was reduced by 0.5-fold (Fig. 2B), which is indicative of reduced euchromatin formation in arsenic exposed differentiating C2C12 cells.

***Arsenic exposure increases Ezh2 nuclear expression and recruits Ezh2 to the myogenin promoter at the transcription start site.***

The polycomb Ezh2 methyltransferase (Ezh2) has been reported to play a role in the repression of terminal muscle differentiation by epigenetic mechanisms (Caretti et al. 2004; Juan et al. 2009). Because histone marks were changed by arsenic exposure on the myogenin promoter at the TSS, we asked whether arsenic exposure would increase Ezh2 expression during muscle differentiation. *Immunofluorescence* staining indicates that the nuclear localization of Ezh2 was significantly increased by ~2-fold in cells treated with 20 nM arsenic (Figs. 3A and 3B). Additionally, results from ChIP assays also showed a significant 3.3-fold recruitment of Ezh2 to the myogenin promoter surrounding the transcription start site in the arsenic-exposed cells (Fig. 3C). These data indicate that Ezh2-mediated gene silencing is increased after arsenic exposure.

***Arsenic exposure recruits Dnmt 3a, but not Dnmt 3b, to the myogenin promoter***

Since Ezh2 can silence gene expression by directly recruiting DNA methyltransferases (Dnmts) to the target gene's promoter (Viré et al. 2006), we examined the recruitment of Dnmt 3a and Dnmt 3b, which are both de novo DNA methyltransferases (Ling et al. 2004), to the myogenin promoter in cells exposed to 20 nM arsenic. Interestingly, the area surrounding the TSS (ChIP 3) showed a significant 1.9-fold enrichment in Dnmt3a in arsenic exposed cells (Fig. 4A). However, there was no significant difference in the recruitment of Dnmt 3b to the myogenin promoter between control and arsenic treatments (Fig. 4B).

***Arsenic exposure reduces the recruitment of Mef2 to the myogenin promoter***

MyoD and Mef2 are two transcription factors that activate myogenin expression by recruiting CBP/p300 co-activator proteins to the myogenin promoter. These co-activators possess histone acetyltransferase (HAT) activity, which results in the relaxation of chromatin structures (Lu et al. 2000; Ohkawa et al. 2006). Our previous study showed a significant reduction in Mef2 nuclear translocation in arsenic-treated C2C12 cells during differentiation (Steffens et al. 2011) and we wanted to determine whether this reduced nuclear translocation was due to reduced recruitment of Mef2 to the myogenin promoter. There is one Mef2 response element in this region (Fig. 2A), so only the primers specific for –31 to –128 (ChIP 2) were used. Mef2 recruitment was indeed reduced by ~70% on the myogenin promoter after arsenic exposure at DM2 (Fig. 5A). The nuclear expression of MyoD was also quantified by *immunofluorescence*, but there

was no change in its expression during the differentiation of arsenic-exposed C2C12 cells (Figs. 5B and 5C).

**Discussion**

Results from the present study, using chromatin immunoprecipitation and *immunofluorescence* staining, illustrate that repressed myogenin expression in arsenic-exposed C2C12 cells is likely due to a combination of reduced Igf-1 expression, enhanced nuclear expression and promoter recruitment of Ezh2, and the altered histone remodeling status on the myogenin promoter (-40 to +42). To our knowledge, this may be the first report that illustrates the effects of nanomolar arsenic concentration on the expression of Ezh2 and Igf-1 on skeletal muscle development. Additionally, this may also be the first study that has examined the effects of nanomolar arsenic concentration on the histone remodeling status at a gene-specific promoter, since previous, studies have examined the effects of arsenic on global histone modifications (Chu et al. 2011; Ramirez et al. 2008; Zhou et al. 2009).

***Reduced Igf-1 expression and its role in myogenin expression.***

Igf-1 is considered to be a myogenic growth factor (Chakravarthy et al. 2000). Results from control C2C12 cells indicate that nuclear Igf-1 expression was increased in a time-dependent manner at DM1 and DM1.5. Two days after the induction of muscle differentiation, the levels of Igf-1 were maximal and the protein was diffusely expressed within the myotubes. However, in arsenic-exposed C2C12 cells, Igf-1 protein and mRNA expression were significantly reduced. Additionally, the expression of myostatin, which represses muscle differentiation by antagonizing the Igf-1/PI3K/Akt pathway (Yang et al.

2007), was modestly increased in the nuclear fraction of the arsenic-exposed C2C12 cells (data not shown). Therefore, the increased nuclear Igf-1 expression may play a role in initiating skeletal muscle differentiation. Indeed, the translocation of Igf-1 into the nuclei has been reported in regenerating human muscle satellite cells, in which Igf-1 protein was detected in the nuclei at 24 hours after the start of regeneration, and then was diffusely expressed in the myofibres after 72 hours, while myogenin mRNA were significantly increased by 2-fold at 24h and ~3-fold after 72 hours (McKay et al. 2008). In contrast, mice lacking Igf-1 have reductions in the number of formed muscle fibers and myogenin expression is significantly lowered (Miyake et al. 2007).

Although the molecular mechanisms about how Igf-1 transcriptionally induces myogenin mRNA are not fully understood, it has been suggested that IGF1/PI3K/AKT target multiple nuclear factors that bind Mef2 and MyoD to PI3K/Akt-responsive elements residing within the 133-bp proximal myogenin promoter (Xu and Wu 2000). Moreover, inhibition of Igf-1 expression in C2C12 cells represses myogenin expression due to recruitment of the polycomb protein Ezh2 and the formation of hypoacetylated and hypermethylated histones on the myogenin promoter between -106 to +91, all of which act to form closed chromatin structures (Serra et al. 2007). In our arsenic-exposed C2C12 cells, we observed the reduction of Igf-1 expression, as well as repressed Mef2 recruitment on the myogenin promoter. Additionally, Ezh2 enrichment, increases in histone marks indicative of heterochromatin and a reduction in histone marks indicative of euchromatin were detected on the myogenin promoter at -40 to +42 following arsenic

exposure. Therefore, our results indicate that nanomolar concentrations of arsenic repress myogenin expression through Igf-1-mediated regulatory mechanisms.

***Arsenic exposure modifies histones near the TSS in the myogenin promoter.***

The expression of muscle genes during myoblast differentiation is regulated by chromatin structure, in which heterochomatin is present at muscle-regulatory elements in undifferentiated myoblasts and then as the myoblasts enter the differentiation process, the chromatin structure becomes permissive (Guasconi and Puri 2009; Saccone and Puri 2010). Our ChIP assays indicate that arsenic-exposed C2C12 cells have a 3-fold increase of repressive chromatins (H3K9 Me2 and H3K9 Me3), and a 50 % reduction of permissive chromatins (H3K9 Ac) at the TSS (-40 to + 42) of the myogenin promoter when they should be undergoing differentiation. This area of increased heterochomatin is at the 133-bp proximal myogenin promoter region, and encompasses a TATA box, a MyoD site (E-box), and the transcription start site (Buchberger et al. 1994; Yee and Rigby 1993). The proximal myogenin promoter has been identified as a critical regulatory element for myogenin expression (Buchberger et al. 1994; Deato and Tjian 2007; Yee and Rigby 1993). For example, it has been demonstrated that the binding of TRF3/TAF3 (TBP-related factor 3/TATA-binding protein-associated factors 3) to the TATA box and recruitment of RNA Pol II-ser5 to the proximal promoter are both required to activate the myogenin promoter in differentiating C2C12 cells (Deato and Tjian 2007). Therefore, the altered chromatin conformation by arsenic exposure may affect the accessibility of basal transcription factors to the proximal promoter and thereby result in the reduction of myogenin in differentiating C2C12 cells exposed to arsenic.

To date, several metals have been reported to repress the basal machinery *via* histone remodeling on promoter regions of some genes. For instance, in mouse Hepa-1 cells, chromium blocks the transcription of the *Cyp1a1* gene by inhibiting RNA polymerase II recruitment to the proximal promoter through the reduced acetylation of H3K9 (Schnekenburger et al. 2007). In human lung carcinoma A549 cells and normal bronchial epithelial BEAS-2B cells, chromate silences the expression of the tumor suppressor gene MLH1 *via* induction of H3K9 methylation in its promoter (Sun et al. 2009). Moreover, nickel silences the gpt (bacterial xanthine guanine phosphoribosyltransferase) transgene in G12 Chinese hamster cells due to increased H3K9 Me2 enrichment (Chen et al. 2006). Therefore, these and other results suggest that arsenic-mediated histone modifications at the myogenin proximal promoter may play a potential regulatory role in reducing myogenin expression.

***Induced EZH2 expression and its potential role in repressing myogenin expression***

A previous report indicated that increased Ezh2 expression correlates to reduced myogenin expression, and silenced Ezh2 expression induces myogenin expression in differentiating C2C12 cells (Juan et al. 2009). Consistently, in our arsenic-exposed C2C12 cells, which have been shown to exhibit delayed muscle differentiation due to reductions in myogenin expression (Steffens et al. 2011), the nuclear expression of Ezh2 was significantly enhanced by 2-fold. Therefore, Ezh2 seems to be one of the potential mechanisms involved in abnormal muscle development due to arsenic. Indeed, Ezh2 has been reported as a negative muscle regulator. For example, in dystrophic muscles, muscle regeneration is inhibited by recruitment of Ezh2 and Dnmt3b in muscle satellite

cells, which thereby represses Notch-1 expression (Acharyya et al. 2010). Interestingly, results from our ChIPs also demonstrate a significant increased recruitment of Ezh2 (3.3-fold) and Dnmt3a (~2-fold) to the myogenin promoter at the TSS in arsenic–treated cells. Recruitment of Dnmt3a to the myogenin promoter at the TSS may be a reason why 55% of the CpGs at +3 on the myogenin promoter were methylated in arsenic treatments (Steffens et al. 2011). Such a high methylation rate of CpG at +3 may alter myogenin expression. For example, a recent study has shown that CIBZ, a methyl-CpG-binding protein, suppresses muscle development by directly binding to the myogenin proximal promoter and thus inhibiting myogenin expression (Oikawa et al. 2011). Luo and coworkers have demonstrated, using C2C12 cells, that methyl-CpG-binding protein 2 (MCB2) suppresses muscle terminal differentiation by inducing heterochromatin formation (H3K9 Me2) at the myogenin proximal promoter (Luo et al. 2009). Therefore, these results suggest that the enriched Dnmt3a after arsenic exposure may highly methylate CpG site at +3, thereby recruiting the methyl-CpG-binding proteins, which results in the silencing of the myogenin gene by increasing heterochromatin formation surrounding the TSS of myogenin.

Since Ezh2 has been reported to silence myosin heavy chain (MHC) and muscle creatine kinase (MCK) by enzymatic methylation of H3K27 (Caretti et al. 2004), we also examined the tri-methylation of histone H3K27 on myogenin promoter at TSS. However, there was only a 1.6-fold induction of H3K27 Me3 at TSS in arsenic treatments (data not shown). Therefore, it appears that the induced Ezh2 expression may repress myogenin expression through recruiting Dnmt3a, rather than performing histone H3K27

trimethylation, on myogenin promoter at TSS in arsenic treatments. To date, the regulatory mechanisms about how arsenic exposure induces Ezh2 expression remain largely unclear. Thus, further examinations of arsenic-mediated Ezh2 expression are required in the future.

During muscle differentiation, the methylation status of the myogenin promoter correlates with myogenin expression (Lucarelli 2000; Palacios et al. 2010). After arsenic treatment of C2C12 cells, two hypermethylated CpGs at -236 and -126 and a hypomethylated CpG at -207 were detected (Steffens et al. 2011). Recently, two muscle transcription factors, SP1 (trans-acting transcription factor 1) and Snail (Snail homolog 1, Drosophila), have been predicted to play a role in myogenin expression by binding to myogenin promoter at sites -234 to -222 for SP1 and -207 to -201, for Snai1 (Fuso et al. 2010). We did performed DNA-protein pull down and ChIP assays in differentiating C2C12 cells treated with or without 20nM arsenic, but, no differences in DNA-protein binding of SP1 and Snail on the myogenin promoter was detected between control and arsenic groups (data not shown). Therefore, the reasons for and the consequence of these arsenic-induced abnormal CpGs in the myogenin promoter remain unclear.

In conclusion, our results indicate that 20 nM sodium arsenite reduces Igf-1 expression in differentiating C2C12 cells. Such decreased Igf-1 may be responsible for the reduced recruitment of Mef2 on the myogenin promoter, thereby decreasing myogenin expression after arsenic exposure. Additionally, arsenic exposure to C2C12 mouse myocyte cells alters histone remodeling status on the myogenin promoter surrounding the transcription start site, which may reduce the accessibility of basal

transcription factors and repress myogenin expression. Moreover, the nuclear expression of Ezh2, which is known as a negative muscle regulator, was enhanced by arsenic exposure in C2C12 cells. Collectively, we conclude that, rather than acting alone, these altered regulatory mechanisms by arsenic exposure seem to be connected and co-contribute to the repressed myogenin gene in C2C12 cells.

**Acknowledgements**

We thank Terry Bruce for her help with the immunofluoresence assays. Funding for this study was provided by NIH (ES016640 and ES016640-1S1).

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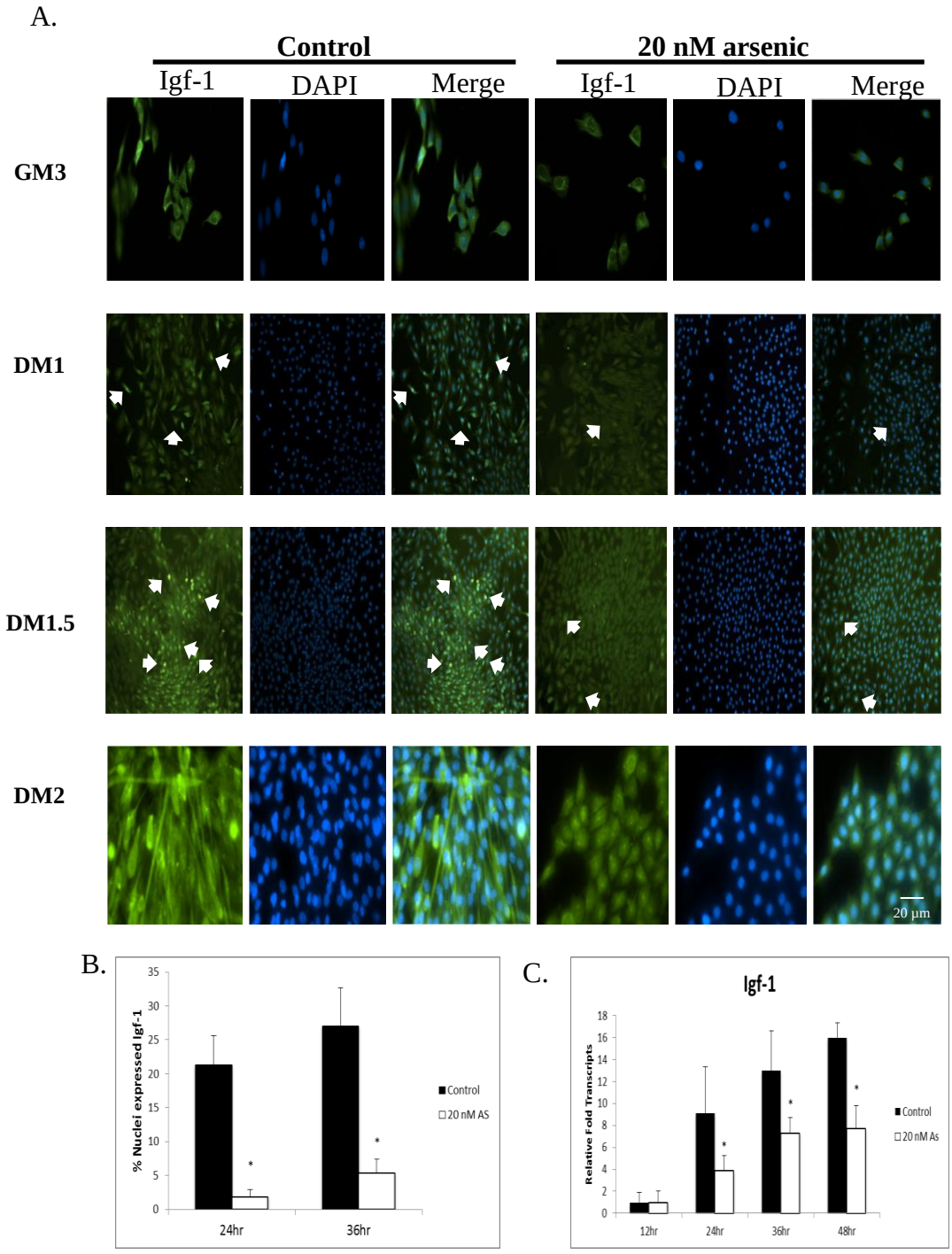



**Fig. 3.1 Arsenic exposure reduces Igf-1 expression and nuclear translocation in C2C12 cells.** C2C12 cells were cultured with or without 20nM sodium arsenite in growth medium for 3 days (GM3) and then differentiation medium (DM) for 1, 1.5, or 2 days to

examine Igf1 expression by immunofluorescence. Arrows indicates nuclei expressing Igf-1 (A). The percentage of nuclei expressing Igf-1 was determined by randomly selecting 6 fields per chamber, for a total of four chambers/time point/group. Results were averaged and statistical differences (*) determined by Student's *t*-test ($p<0.05$) (B). Igf-1 mRNA expression from C2C12 cells in the presence and absence of 20 nM arsenic in differentiation medium for 12 hours, 24 hours, 36 hours, and 48 hours was quantified by qPCR. Each sample was run in triplicate (n=3 plates/day/group) and results were normalized to GAPDH and expressed as normalized fold-change to relative to C2C12 12hr control) cells. Statistical differences (*) were determined by Student's *t*-test ($p<0.05$) (C).

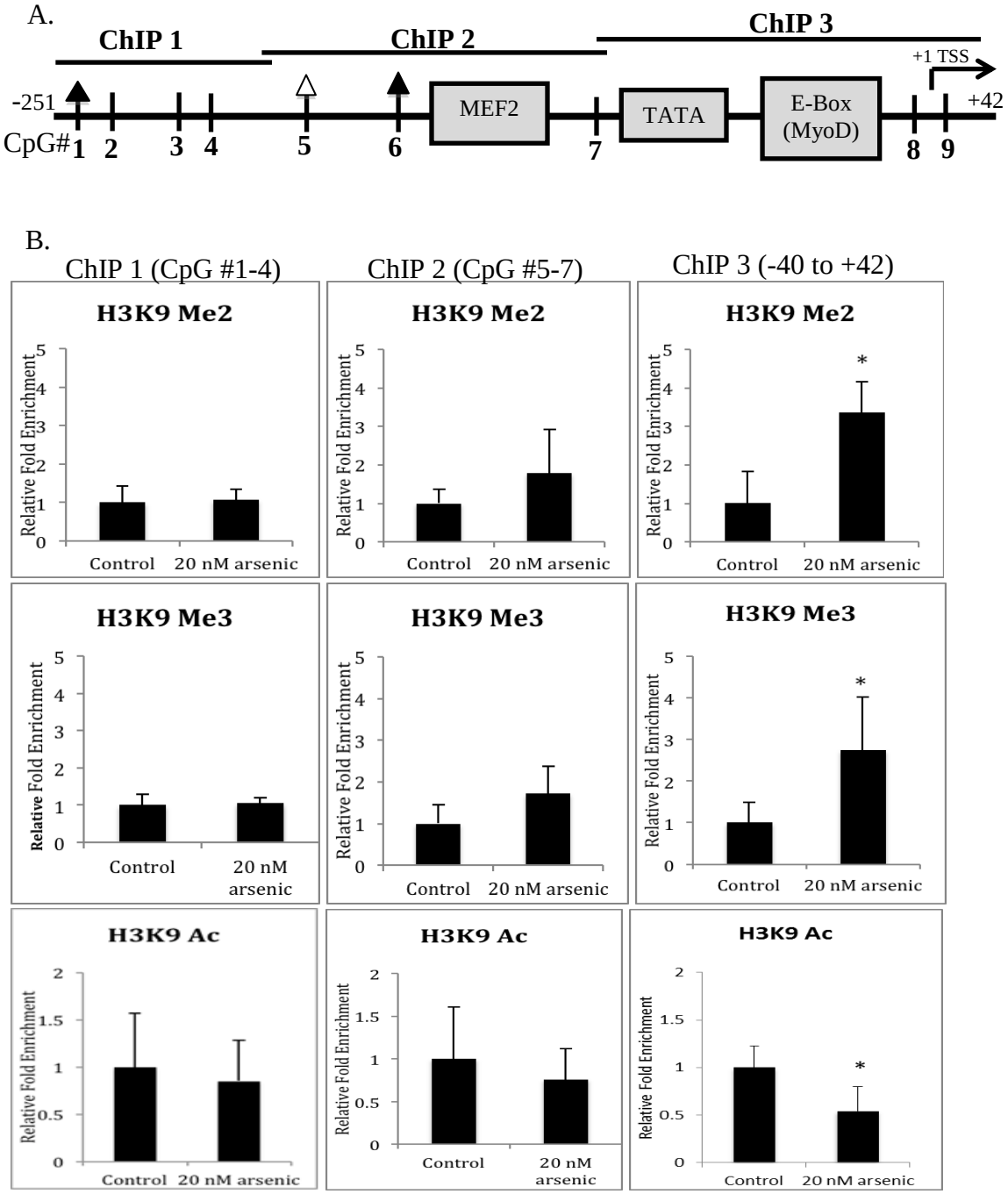



**Fig. 3.2 Arsenic exposure alters the histone remodeling status of the myogenin promoter in C2C12 cells.** Schematic diagram of the myogenin promoter, showing the transcription start site (TSS), Mef2 response element, TATA box, E-box (MyoD binding

site), CpG sites, and the locations where the three ChIP assays were performed. Black triangles represent two hypermethylated CpGs at -236 and -126, whereas the white triangle represents a hypomethylated CpG at -207 on myogenin promoter after sodium arsenite exposure to differentiating C2C12 cells (Steffans et al. 2011) (A). ChIP assays were performed with antibodies against di-methylated H3K9 (H3K9 Me2), tri-methylated H3K9 (H3K9 Me3), and acetylated H3K9 (H3K9 Ac) using DNA harvested from DM2 C2C12 cells exposed to 0 or 20nM sodium arsenite. Enriched DNA fragments from these ChIP assays were analyzed by qPCR and expressed as relative fold enrichment. Each sample was ran in triplicate (n=4 plates/day/group) and statistical differences (*) were determined by Student's t-test ($p<0.05$) (B).

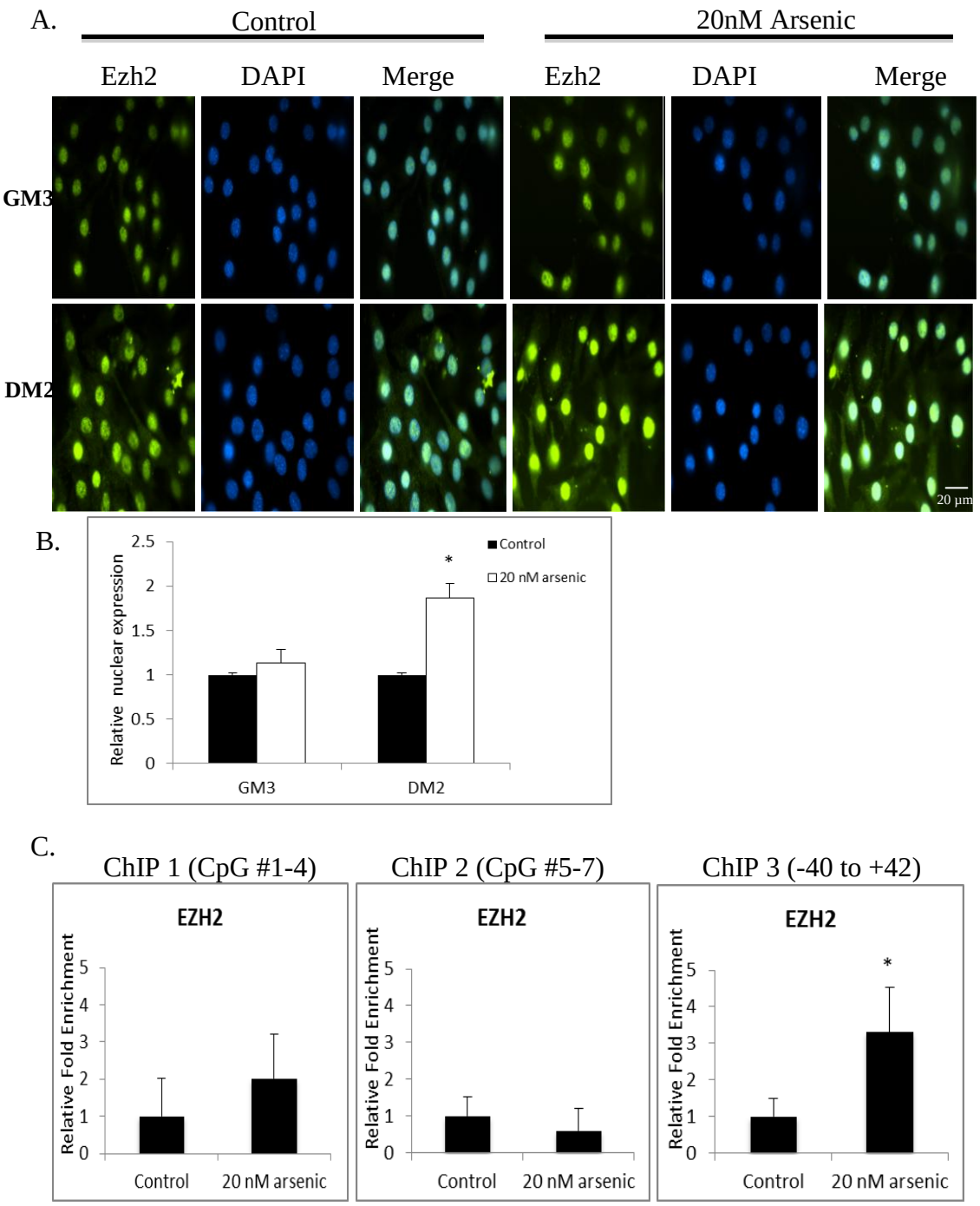
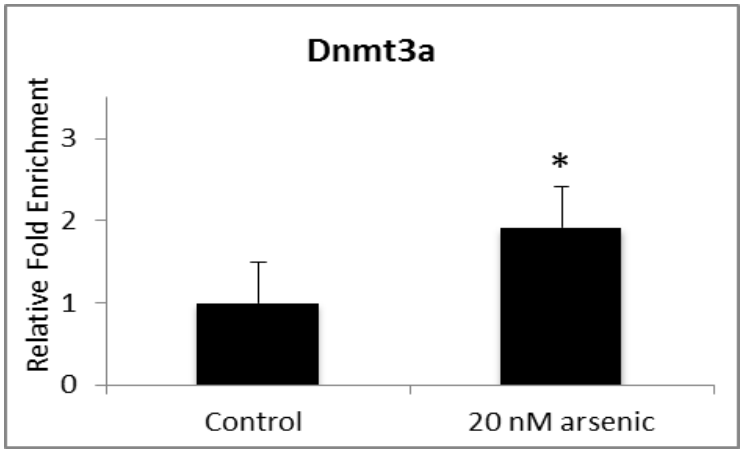



**Fig. 3.3 Arsenic exposure enhances Ezh2 nuclear expression and recruits Ezh2 to the myogenin promoter near the transcription start site.** Ezh2 protein expression was examined by immunofluorescence in C2C12 cells exposed with or without 20nM arsenic in the growth medium for 3 days (GM3) and in the differentiation medium for 2 days (DM2). Pictures are representative examples from 4 independent wells/time point/group

(A). Differences in Ezh2 protein expression between control and arsenic-treated cells were determined by measuring the nuclear intensiy in 6 randomly selected fields per chamber, for a total of four independent experiments/time point/group. Intensities are expressed as relative nuclear expression (B). ChIP was performed with antibodies against Ezh2 using C2C12 cells at DM2. Immunoprecipitated DNA was analyzed by qPCR and the data is expressed as relative fold enrichment (C). Each sample was run in triplicate (n=4 plates/day/group) and statistical differences (*) were determined by Student's t-test ($p<0.05$).

A.




B.

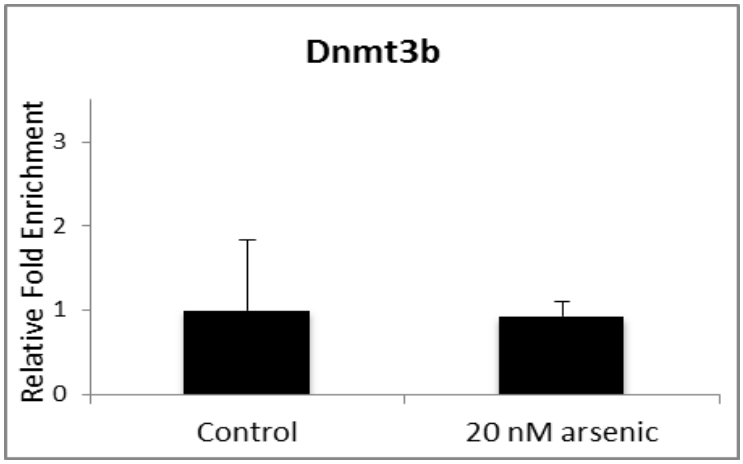



**Fig. 3.4 Ezh2 recruits Dnmt 3a, but not Dnmt 3b, to the myogenin promoter.** ChIP was performed at the TSS (-40 to +42) of the myogenin promoter using antibodies against Dnmt 3a (A) and Dnmt 3b (B) with DNA from C2C12 cells at DM2 exposed to 0 or 20nM arsenic. Enrichment of Dnmts was examined by qPCR and data are expressed as relative fold enrichment. Each sample was run in triplicate (n=7 plates/day/group) and statistical differences (*) were determined by Student's t-test ($p<0.05$).

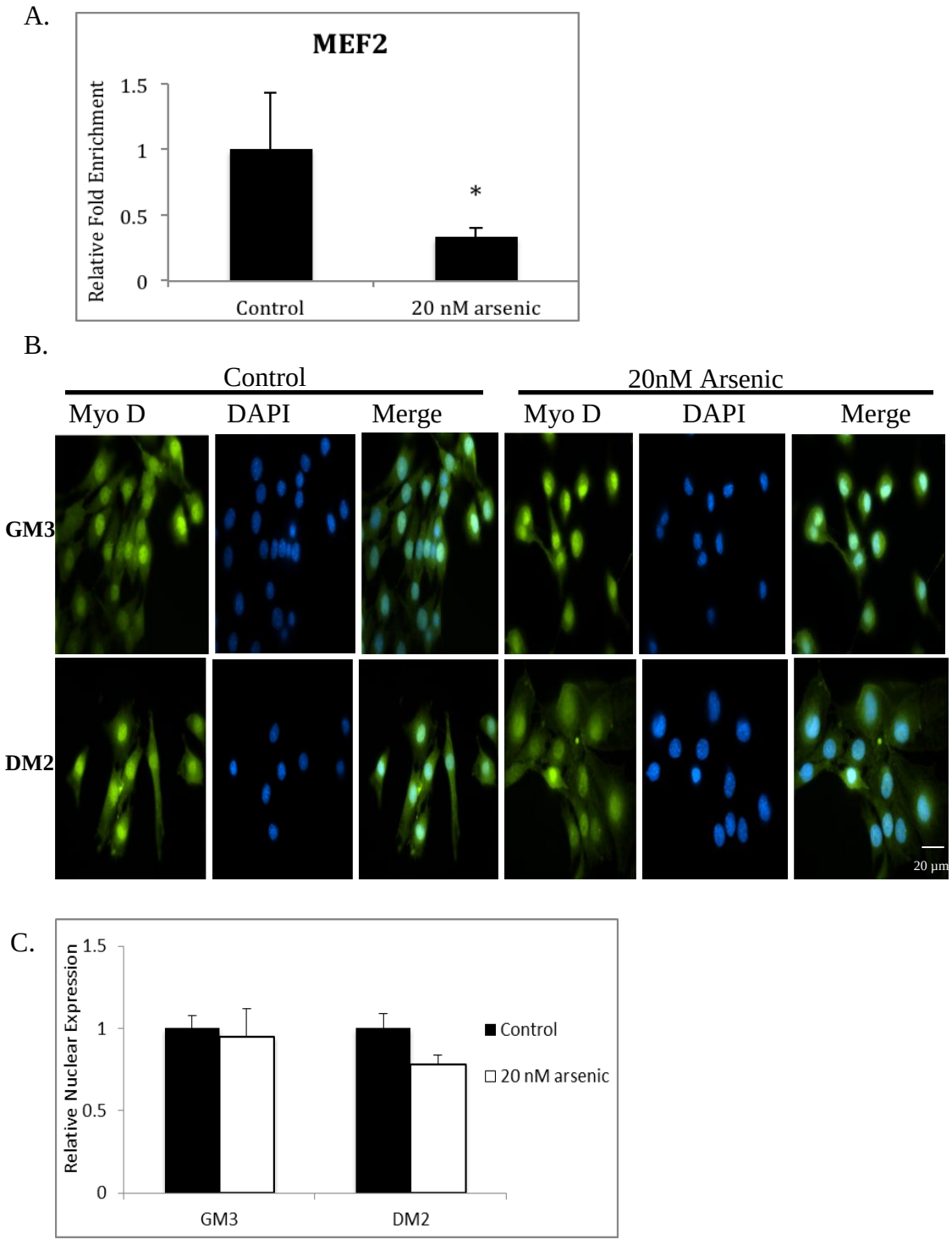



**Fig. 3.5 Arsenic exposure reduces the recruitment of Mef2 to the myogenin promoter but does not alter MyoD expression.** On DM2 in cells cultured with or without 20nM arsenic, ChIP was performed at the ChIP 2 region with antibodies against

Mef2. Enriched DNA fragments were analyzed by qPCR and are expressed as relative fold enrichment (A). MyoD expression in C2C12 cells exposed with or without 20nM arsenic at GM3 and DM2 was examined by immunofluorescence (B). MyoD protein expression was quantified by measuring nuclear intensities in 6 randomly selected fields per chamber, for a total of four chambers/time point/group. Intensities were expressed as relative nuclear expression (C). Each sample was ran in triplicate (n=4 plates/day/group) and statistical differences (*) were determined by Student's *t*-test ($p<0.05$).

CHAPTER FOUR

# ARSENIC EXPOSURE INHIBITS MYOGENESIS AND NEUROGENESIS IN P19 STEM CELLS THROUGH β-CATENIN REPRESSION

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This manuscript is to be submitted to *Toxicological Sciences* and is in the format required of that journal.

**Abstract**

Epidemiological studies have correlated arsenic exposure with adverse developmental outcomes such as stillbirths, neonatal mortality, and low birth weight. Additionally, arsenic-induced effects on neuronal formation and function, such as reduced intellectual function and reductions in neuronal cell migration and maturation, and changes in skeletal muscle cell formation, alteration of muscle fiber subtype, and changes in locomotor activity, have been reported. The current study used P19 mouse stem cells to examine whether arsenic exposure could alter their differentiation into skeletal muscle and neurons. When P19 cells were exposed to 0.5μM sodium arsenite, their differentiation into embryoid bodies was not altered. However, arsenic suppressed their differentiation into muscles and neurons, as evidenced by morphological changes accompanied by a significant reduction in myosin heavy chain and Tuj1 expression. Real time PCR, immunofluorescence, and immunoblotting were used to confirm that the altered differentiation was due to the repression of the muscle-and neuron-specific transcription factors such as Pax3, Myf5, MyoD, myogenin, neurogenin 1, neurogenin 2, and NeuroD in the arsenic-exposed cells. The reductions in transcription factors expression appear to be caused by repressed Wnt/β-catenin signaling pathways in early embryogenesis, as evidenced by decreased β-catenin expression in the arsenic-exposed embryoid bodies on differentiation days 2 and 5. This study demonstrates that arsenic can disturb the embryonic differentiation process by repressing the Wnt/β-catenin signaling pathway. More importantly, this study may provide insight into how arsenic exposure affects skeletal and neuronal differentiation during embryogenesis.

**Keywords:** Sodium Arsenite, P19, stem cells, Beta-Catenin, myogenesis, neurogenesis

## Introduction

Exposure to arsenic in drinking water is a global health issue affecting millions of people (Cherry et al., 2008; Medrano et al., 2010; Mohammad et al., 2009) and it has been associated with a variety of adverse effects including cancer, skin lesions, and cardiovascular disease (Benbrahim-Tallaa & Waalkes, 2007; Schuhmacher-Wolz et al., 2009; States et al., 2009). Arsenic can also act as a developmental toxicant. In humans and mice, arsenic can pass through the placental barrier (Concha et al., 1998; Jin et al., 2006; Xie et al., 2007) and, *in utero* exposure of arsenic *via* the mother's drinking water can result in increases in neonatal death and miscarriages, and reduced birth weight (Agusa et al., 2010; Concha, et al., 1998; Markowski et al., 2011; Raqib et al., 2009; von Ehrenstein et al., 2006).

Additionally, arsenic can cross the blood-brain barrier (BBB) and accumulate in the brain (Au et al., 2008; Jin, et al., 2006; Kiguchi et al., 2010; Knipp et al., 2007; Xi et al., 2010), which may provide a rationale for the correlations between embryobnic arsenic exposure and neurological diseases, such as mental retardation, hearing impairment, and lower intelligence quotient scores (Bencko et al., 1977; Calderon et al., 2001; Dakeishi et al., 2006; Tsai et al., 2003). Arsenic exposure can.inhibit neurite outgrowth in Neuro-2a (N2a) cells by blocking the activation of adenosine monophosphate-activated kinase (AMPK) pathway (Wang et al., 2010). In primary embryonic rat neuroepithelial cells, arsenic inhibits cell cycle progression in all cell cycle phases, and the arrested cell cycle phases were associated with the induced apoptosis (Sidhu et al., 2006). Piao and coworkers have observed that arsenic exposure *via* drinking

water induces degeneration of neuronal cells in the cerebrum and cerebellum of mice (Piao et al., 2005). Moreover, in rats, oral arsenic administration resulted in thinly myelinated axons in peripheral sensory nerves, caused by the induction of reactive oxygen species (Garcia-Chavez et al., 2007).

Arsenic-mediated adverse effects on muscle differentiation have also been reported. For example, arsenic exposure to mouse C2C12 myoblasts resulted in delayed muscle differentiation into myotubes, due to a reduction in the expression of myogenin (Steffens et al., 2011). In rodent models, arsenic suppresses the regeneration of injured muscles (Yen et al., 2010), alters pulmonary structure and function *in utero* by increasing the smooth muscle actin in the lung (Lantz et al., 2009), and disrupts the smooth muscle integrity around the blood vessels in the heart (Hays et al., 2008) and the thoracic aorta (Lim et al., 2011).

Additionally, rats exposed to arsenic in *utero* had reduced locomotor activity and reductions in limb movements (Chattopadhyay et al., 2002; Rodríguez et al., 2002). Collectively, these results suggest that arsenic acts as a developmental toxicant by affecting the development of the musculature and neurons. However, the molecular mechanisms responsible these multiple adverse outcomes remain largely unknown.

Embryonic stem cells (ECSs) are pluripotent cells capable of differentiation into multiple cell lineages (Angello et al., 1997; Vanderheyden & Defize, 2003; Wobus & Guan, 1998). Since these cells recapitulate gene expression patterns during early embryogenesis (Marikawa et al., 2009; Rohwedela et al., 2001), the use of stem cells is a promising *in vitro* model to assess developmental toxicity (Adler et al., 2008; Bal-Price et

al., 2010). To this end, it has been shown that exposure of 76 nM arsenic to human stem cells caused a significant down regulation of genes indicative of all the three germ layers (Flora & Mehta, 2009). Results from mouse embryonic stem cells indicate that 10 mM arsenic reduces the formation of embryoid bodies and thereby inhibits cardiac cell differentiation (Stummann et al., 2008). Moreover, Tokar and coworkers have demonstrated that 5μM arsenic can transform human epithelial stem cells into a pluripotent cancer stem cell phenotype (Tokar et al., 2010). However, the molecular mechanisms responsible for arsenic's effects during embryogenesis are not well understood.

The Wnt/ β-catenin signaling pathway plays an important role in somite formation and neural crest development (Borello et al., 2006; Burstyn-Cohen et al., 2004; Geetha-Loganathan et al., 2008; Huelsken & Birchmeier, 2001; Schmidt et al., 2008). In stem cells, β-catenin regulates self-renewal and cell fate decisions (Ling et al., 2009; Liu et al., 2008; Lyashenko et al., 2011). For example, β-catenin-deficient mouse stem cells self-renew rather than differentiate into the three germ layers (Lyashenko et al., 2011), while overexpression of β-catenin alone triggers stem cells to differentiate into muscles (Petropoulos & Skerjanc, 2002) and neurons differentiation in the WWE6 ES cells (Otero et al., 2004).

Mouse P19 cells are pluripotent embryonal carcinoma cells, which can differentiate into multiple cell types under the appropriate conditions (Macpherson & McBurney, 1995; McBurney, 1993). Treatment with dimethyl sulfoxide (DMSO) induces P19 cells to differentiate into mesoderm and endoderm lineage, while retinoic acid

exposed P19 cells differentiate into neuroectoderm lineage (Macpherson & McBurney, 1995). P19 cells can grow continuously in serum-supplemented media without feeder cells, which makes them a good model to investigate the impacts of arsenic exposure on cellular differentiation. The goal of this study was to determine if arsenic reduced myogenesis and neurogenesis due to altered Wnt/ β-catenin signaling in embryoid bodies.

**Methods**

*P19 cell culture and differentiation*

The mouse embryonal carcinoma P19 cell line (ATCC, Manassas, VA) was maintained in α-MEM supplemented with 7.5% bovine calf serum (Hyclone, Logan, UT), 2.5% fetal bovine serum (Mediatech, Manassas, VA), 1% L-glutamine, and 1% penicillin/streptomycin (designated as growth medium) at 37°C in a humidified incubator containing 5% $CO_2$. Medium renewal was conducted every 48 hours. To induce differentiation, P19 cells were aggregated by the hanging drop method (Wang & Yang, 2008) with some modification. Briefly, P19 cells were trypsinized and suspended in differentiation medium (growth medium containing 1%DMSO) with or without 0.5μM sodium arsenite at a density of 500cells/ 20μl or drop. Ninety-six drops of cell suspension were placed on the up-turned inner surface of the lid of a 150mm petri dish, which was inverted and placed on top of the dish containing 10ml of PBS, and incubated for 2 days (day 2). After 2 days, each individual drop was transferred to a 96-well ultralow attachment plate containing 70μl of fresh differentiation medium with or without 0.5μM arsenite. After 3 days (day 5), the embryoid bodies were transferred to a 0.1% gelatin coated 48-well plate containing 300μl of fresh differentiation medium with or without

0.5μM sodium arsenite. The medium was renewed every 48 hours until cells were harvested.

*qPCR*

P19 cells were cultured with or without 0.5μM sodium arsenite as described above. When harvesting aggregates on day 5 culture, embryoid bodies from a 96-well plate were collected and combined as one replicate (n=3 per group per day), while for day 9 collection, cells from all wells in a 48-well plate were trypsinized and combined as one replicate (n=3 per group per day). Total RNA were extracted using TRI reagent (Sigma-Aldrich, St. Louis, MO) according to the manufacturer's instruction. RNA (2μg) was reverse transcribed into cDNA and the expression of paired box 3 (Pax3), paired box 7 (Pax7), myogenic factor 5 (Myf-5), MyoD, myogenin (Mgn), neurogenin-1 (Ngn-1), neurogenin-2 (Ngn-2), NeuroD, and Gapdh was quantified by qPCR using SYBR Green (SABiosciences, Frederick, MD) and the appropriate primers (Pax3 Forward 5'-CCT CTG CCC AAC CAT ATC CG-3', Reverse: 5'-GAA ATG ACG CAA GGC CGA ATG-3'; Pax7 Forward: 5'-TCT CCA AGA TTC TGT GCC GAT-3', Reverse: 5'-CGG GGT TCT CTC TCT TAT ACT CC-3'; Myf-5 Forward: 5'-TGC TGT TCT TTC GGG ACC AGA CAG G-3', Reverse: 5'-GGA GAT CCT CAG GAA TGC CAT CCG C-3'; MyoD Forward: 5'-ATG CTG GAC AGG CAG TCG AGG C-3', Reverse: 5'-GCT CTG ATG GCA TGA TGG ATT ACA-3'; Mgn Forward: 5'- CCA ACC CAG GAG ATC ATT TG-3', Reverse: 5'- ACG ATG GAC GTA AGG GAG TG-3'; Ngn-1: Forward 5'-CCA GCG ACA CTG AGT CCT G-3', Reverse: 5'-CGG GCC ATA GGT GAA GTC TT-3'; Ngn-2: Forward 5'-AAC TCC ACG TCC CCA TAC AG-3', Reverse: 5'-GAG GCG CAT

AAC GAT GCT TCT-3'). Gapdh was used as the housekeeping gene (Gapdh Forward: 5'-TGC GAC TTC AAC AGC AAC TC-3', Reverse: 5'-ATG TAG GCC ATG AGG TCC AC-3'). Samples were run in triplicate, and relative gene expression was calculated using the comparative threshold (Ct) method (Livak & Schmittgen, 2001). The experiments were replicated at least three times. The results were expressed as mean ± SD (standard deviation). Student's *t*-test was used to determine significant differences ($p<0.05$) between control and arsenic groups.

*Immunohistochemistry and immunoblotting of differentiated cells*

Embryoid bodies exposed with or without 0.5μM sodium arsenite were prepared as described above. On day 5, embryoid bodies were plated onto 10-cm tissue culture dishes containing 0.1% gelatin coated coverslips. Medium renewal was conducted every 48 hours until the day for immunofluorescence (n=5 per group per day). Cells were fixed with methanol at -20°C for 5 minutes, blocked in 1% bovine serum albumin, 0.1% Triton-X100 in PBS, and incubated with the appropriate primary antibody for 1 hour (β-catenin: 1:100 dilution, Gene Tex # GTX101254; Pax3: 1:200 dilution, Gene Tex #GTX100663; MyoD: 1:100 dilution, Santa Cruz # SC-304; Tuj1: 1:100 dilution, Millipore # MAB1637; and myosin heavy chain: 50 μl of antibody supernatant, DSHB# MF20). The secondary antibody (1μg/mL) conjugated to Alexa Flour 488 (Invitrogen, Carlsbad, CA) was incubated with the cells, which were counterstained with DAPI (Invitrogen). Cells were examined by conventional immunofluorescence on a Ti Eclipse Inverted Microscope (Nikon, Melville, NY).

Protein extractions and immunoblots were performed according to standard methods (Dignam et al., 1983; Towbin et al., 1979). When harvesting aggregates on day 5 culture, embryoid bodies from a 96-well plate were collected and combined as one replicate (n=3 per group per day), while for day 12 collection, cells from all wells in a 48-well plate were trypsinized and combined as one replicate (n=3 per group per day). The same primary antibodies were used as for the immunofluorescence (Pax3: 1:1000 dilution; Tuji: 1:100 dilution; myosin heavy chain: 1:50 dilution). Anti-Tbp antibody (1:1000 dilution, Abcam#818) and anti-Gapdh antibody (1:1000 dilution, IMGENEX# IMG-5019A-1) were used as loading controls.

*Immunohistochemical analysis of embryoid bodies*

Embryoid bodies at day 2 and day 5 were exposed to 0 or 0.5μM arsenite as described above. For day 2 collection, ninety-six drops of cell suspension were harvested from a 150mm petri dish combined as one replicate (n=3 per group per day), while for embryoid bodies on day 5 culture, aggregates from a 96-well plate were collected and combined as one replicate (n=3 per group per day). Once harvested, embryoid bodies were fixed for 24 hours in 10% neutral buffered formalin, dehydrated in graded ethanol, processed, and embedded in paraffin. Sections (5μm) were placed on slides, deparaffinized with xylene, and rehydrated in graded ethanol solutions. β-catenin and Pax3 staining was carried out in a dark humid chamber for 1 hour, using anti-β-catenin antibody (1:100 dilution, Gene Tex # GTX101254) and anti-Pax3 antibody (1:200 dilution, Gene Tex #GTX100663). The secondary antibody (1μg/mL) conjugated to Alexa Flour 488 (Invitrogen, Carlsbad, CA) was incubated with the slides, which were

counterstained with DAPI (Invitrogen). Slides were examined by conventional immunofluorescence on a Ti Eclipse Inverted Microscope (Nikon, Melville, NY).

## Results

### *Arsenic represses skeletal muscles and neuronal differentiation in P19 cells*

To examine the developmental effects of arsenic exposure on stem cell differentiation, P19 cell aggregates (embryoid bodies, EBs) were formed in hanging drop culture containing 1% DMSO with or without 0.5μM sodium arsenite. According to our morphological observations, endoderm-like smooth muscles were observed in the differentiating outgrowths from the control embryoid bodies on day 7 (Fig. 1A). Later on, myoblasts and myotubes, representing about 15% of the total cells, could be identified at day 9 (Fig. 1C) and day 12 (Fig. 1E), respectively. These results are consistent with previous reports (McBurney, 1993; Skerjanc, 1999). Interestingly, in our experiments, neurons were also formed at days 9 and 12 (Fig.1E and 1G), representing 30-40% of the total cells by day 12. The morphology of embryoid bodies exposed to 0.5μM arsenic was the same as that from the control groups (Fig. 1B). However, the formation of smooth cells, skeletal muscles, and neurons were significantly reduced (less than 5% of total cells). Instead, the majority of differentiated cells retained the typical epithelial cell-like morphology (~80% of total cells, Fig. 1D, F, and H). A final ~10 to 15% of the total cell numbers in the arsenic-exposed group remained as embryoid bodies, without any sign of differentiation out from the embryoid body (data not shown).

***Arsenic represses β-catenin expression in the differentiating embryoid bodies***

Since Wnt/ β-catenin signaling plays an important role in the induction of early myogenesis and neurogenesis (Marikawa, et al., 2009; Petropoulos & Skerjanc, 2002; Tang et al., 2002), we next asked whether the repressed skeletal muscle and neuronal differentiation in the arsenic-exposed P19-derived embyroid bodies was due to altered Wnt/β-catenin signaling. Immunofluorescence was conducted to examine the expression of β-catenin in embryoid bodies during days 2 and days 5 of aggregation. Results from our control groups indicate that β-catenin was highly expressed in day 2 and then expression levels were reduced in day 5 (Fig 2A and C). However, in arsenic-treated P19 cells, the expression of β-catenin in the embryoid bodies was much lower than controls at both day 2 and day 5 (Fig. 2B and D).

***Arsenic represses Pax 3 expression ay the edges of embryoid bodies***

During embryogenesis, Pax3 is an essential transcription factor that regulates skeletal muscle and neuronal differentiation by targeting myogenic and neurogenic transcription factors (Ichi et al., 2011; Lagha et al., 2008; Ridgeway et al., 2000). Moreover, it has been shown that Wnt/ β-catenin activates Pax3 expression in P19 cells (Marikawa, et al., 2009; Petropoulos & Skerjanc, 2002). To this end, we examined whether repressed β-catenin expression in arsenic-exposed embryoid bodies lead to the reduction of Pax3 expression. In control cells, Pax3 was highly expressed in the embryoid bodies on day 2 and day 5 (Fig. 3A) and continued to express in the cells differentiating out from embryoid bodies on day 9 (Fig 3A). However, in arsenic-exposed groups, Pax3 expression was repressed in the embryoid bodies on day 2 and day 5. And

most importantly, Pax3 protein was almost absent in the day 9 cells (Fig. 3A). Examining Pax3 mRNA levels by qPCR corroborated the immunofluorescence, showing a significant reduction in Pax3 transcripts by 2- to 2.7-fold in the arsenic-treated groups on day 5 and day 9, respectively (Fig. 3B). Additionally, immunoblotting showed a reduction of Pax3 protein expression in the nuclear fraction of the day 5 arsenic-exposed embryoid bodies (Fig. 3C).

***Arsenic represses skeletal muscle differentiation through the repressed myogenic regulatory factors (MRFs)***

The development of skeletal muscle is regulated by several myogenic transcription factors, such as Myf5, Myo D, and myogenin (Yokoyama & Asahara, 2011). In myogenesis, Myf5 and MyoD are early transcription factors that direct the differentiation of muscle progenitors to myoblasts. Later on, both Myf5 and MyoD regulate the expression of myogenin, which induces terminal differentiation by converting myoblasts into myotubes (Carvajal & Rigby, 2010; Gianakopoulos et al., 2011; Yokoyama & Asahara, 2011). Therefore, to examine whether the repressed muscle differentiation by arsenic exposure was due to the reduction of the essential myogenic transcription factors, the expression Myf5, MyoD, and myogenin from P19 cells exposed with or without arsenic during differentiation was determined. In the control groups, Myf5 mRNA transcripts were expressed on day 5 and then were diminished on day 9, MyoD was present on day 5 and its expression was increased by 3-fold on day 9 in the control group. The expression of myogenin was barely detectable on day 5, but it was induced by 150-fold on day 9, as myogenin is needed for terminal muscle differentiation.

In the arsenic-exposed differentiating P19 cells, the expression of Myf5, MyoD, and myogenin in both of the embryoid bodies at day 5 and in the differentiating cells on Day 9 was significantly repressed (Fig. 4A). The transcript results were also seen in protein expression, as immunofluorescence indicated that MyoD expression in the differentiated embryoid bodies was highly repressed in the arsenic groups on day 9 (Fig. 4B). The reductions in myogenin transcription factor expression resulted in suppressed muscle formation, as indicated by the reductions in myosin heavy chain expression on day 12 (Fig. 4C and D).

***Arsenic represses neuronal differentiation through the repressed neurogenic transcription factors***

Neurogenin1, neurogenin 2, and NeuroD are three important transcription factors in neuronal differentiation (Howard, 2005; E. Kim et al., 2011). In mammalians, both neurogenin 1 and neurogenin 2 are expressed in neuronal precursors (neuroblasts) in neurogenesis (Cau et al., 2002; Kim, et al., 2011; Ma et al., 1999), whereas the expression of NeuroD, promotes neuronal fate determination and differentiation (Cherry et al., 2011; Howard, 2005; Ohsawa & Kageyama, 2008). The reduced neuronal differentiation in arsenic-exposed P19 cells is due to a significant reduction in neurogenin 1, neurogenin 2 and NeuroD transcripts on day 5 and day 9 (Fig. 5A), which results in reduced protein expression of the neuronal-specific tubulin Tuj1 on day 12 (Fig. 5B and C).

## Discussion

The results from the present study using P19 stem cells illustrate that sodium arsenite suppresses skeletal muscle and neuronal differentiation through repressed β-catenin expression. Lowered β-catenin levels leads to reductions in the specific transcription factors needed to convert stem cells into neurons and skeletal myotubes. To the best of our knowledge, this is the first study to illustrate that low levels of arsenic can alter the Wnt/β-catenin pathway and repress stem cell differentiation.

To induce P19 differentiation, DMSO has long been used as a chemical reagent for the induction of mesoderm and endoderm lineages (McBurney, 1993; Vanderheyden & Defize, 2003). In this study, surprisingly, we found that DMSO could also induce neuroectoderm lineage in P19 cells. Although reports have demonstrated that DMSO can induce neuronal differentiation in neuroblastoma cells (Kimhi et al., 1976; Oh et al., 2006), this has not been seen in P19 cells. Reports have indicated that, in the absence of retinoic acid, overexpressing neurogenin 1 in P19 cells induces neuronal formation by up-regulating NeuroD expression, (Kim et al., 2004). In the present study, both neurogenin 1 and 2 transcripts were seen during embyroid body formation. In the differentiation and outgrowth period, their expression increased. Neurogenin by 37-fold and 10-fold, respectively expression induces NeuroD (Howard, 2005; Kim, et al., 2004; Osório et al., 2010). NeuroD transcript was barely detectable in the embryoid body, but during the differentiation period, its expression was highly increased, thus leading to neuronal differentiation in P19 cells without any chemical induction. Previous studies have also demonstrated that by co-culturing DMSO-exposed P19 embryoid bodies with

the primitive streak mesoderm-like cell line GCLB, neuronal lineage could be induced successfully without retinoic acid (Pruitt, 1994). The author of that study hypothesized that the neuronal differentiation of P19 cells was due to the synergistic action of a Pax3 signal from GCLB cells and the DMSO treatment (Pruitt, 1994). It is known that Pax3 promotes sensory neuron differentiation in mammalians (Baker et al., 2002; Ichi, et al., 2011; Koblar et al., 1999) and Pax3 can be induced when P19 cells treated with DMSO (Petropoulos et al., 2004; Petropoulos & Skerjanc, 2002). Therefore, it is reasonable to expect that DMSO-induced Pax3 can trigger neurogenin expression in P19 cells.

Our immunofluorescence analysis of sectioned embryoid bodies indicates that β-catenin was expressed on day 2 in the control groups. On day 5, the β-catenin levels were greatly reduced, but remained detectable. Such temporal patterns of β-catenin expression are consistent with previous reports using P19 cells. For example, it has been shown that the level of Wnt/β-catenin reached maximum on day 2 of P19 cell differentiation, during mesoderm formation, and then started to decrease on day 3 (Marikawa, et al., 2009). Moreover, Paige and co-workers have observed that Wnt/β-catenin was expressed on day 2 and then decreased thereafter during cardiac differentiation in human stem cells (Paige et al., 2010). Indeed, in mice, it has been shown that β-catenin is necessary for embryonic development but no longer required for the fetal development, such as the differentiation of myoblasts (Hutcheson et al., 2009). In the arsenic-exposed embryoid bodies, however, β-catenin levels were repressed on day 2 and nearly undetectable on day 5. Such repressed β-catenin indicates that Wnt signaling was reduced in the early stage of embryoid body development by arsenite. This may be the initial regulatory mechanism

responsible for the repressed skeletal muscle and neuronal differentiation on day 9 and day 12 after arsenic exposure. Indeed, it has been shown that β-catenin can directly activate Myf5, as two β-catenin responsive elements in Myf5 promoter are essential for mouse myotomal development (Borello, et al., 2006). Moreover, β-catenin also enhances neurogenesis in neural progenitors through the induction of neurogenin-2 (Hirsch et al., 2007). Our qPCR also shows a significant reduction of Myf5 and Neurogenin-2 expression on day 5 and day 9 upon arsenic exposure.

Recently, altered Wnt/ β-catenin signaling has been reported after arsenic exposure. For example, arsenic trioxide ($As_2O_3$) reduces cytoplasmic β-catenin accumulation and induces apoptosis in human primary myeloma cells (Zhou et al., 2008), while arsenic trichloride ($AsCl_3$)-induced reactive oxygen species (ROS) formation promotes cell transformation and tumorigenesis through the induction of Wnt/ β-catenin signaling in human colorectal adenocarcinoma DLD1 cells (Zhang et al., 2011). Funato and co-workers have found, using HEK293 cells and *Xenopus* experiments, that ROS-mediated β-catenin reduction can be regulated by overexpressing nucleoredoxin, which is a thioredoxin family member (Funato et al., 2006; Funato & Miki, 2010). Since arsenic toxicity has been related to the induction of ROS (Garcia-Chavez et al., 2007; Piao et al., 2005; States et al., 2009), and thioredoxin and nucleoredoxin could act as antioxidant enzymes to protect oxidative stress (Miao & St Clair, 2009; Patenaude et al., 2005), arsenic-induced reactive oxygen species may play a role in the reduction of β-catenin expression in P19 cells.

During differentiation, β-catenin-signaling triggers myogenesis through Pax3 and myogenic regulatory factors, such as Mrf5 and MyoD (Petropoulos & Skerjanc, 2002; Ridgeway et al., 2000). Additionally, β-catenin also promotes sensory neuron differentiation by up-regulating Pax3, (Burstyn-Cohen et al., 2004; Tang et al., 2002). Of these transcription factors, Pax3 is common to both skeletal muscle and sensory neuron differentiation (Howard, 2005; Lassiter et al., 2010; Messina & Cossu, 2009). Therefore, its spatial and temporal expression was examined. As expected, Pax3 expression was lower in the arsenite-exposed embryoid bodies on both day 2 and day 5, likely due to the reduced β-catenin levels. It is interesting that the spatial expression of Pax3 differed between control and arsenite-exposed cells. In control cells, Pax3 is highly expressed at the edges of the embryoid bodies and is later expressed in the differentiating cells on day 9. In the arsenic-exposed cells, Pax3 is expressed in the interior of the embyroid body, but its expression is extremely low at the edges of embryoid bodies. By day 9, Pax3 expression is almost absent in the differentiated regions from arsenic-exposed embryoid bodies. In contrast, on day 9, Pax3 is expressed in the differentiated and differentiating outgrowing cells where the skeletal myotubes and neurons are formed on Day 9 and Day 12.

In the nervous system, Pax3 upregulates Neurogenin 2 (Ichi et al., 2011; Lassiter et al., 2010; Nakazaki et al., 2008), which is a preneuronal gene that plays a key role in the specification of neuronal subtypes, neural crest development, and sensory neurogenesis (Baker, et al., 2002; Dude et al., 2009; Howard, 2005; Nakazaki, et al., 2008). In skeletal muscle progenitors, Pax3 upregulates Myf5, MyoD, and myogenin

(Buckingham, 2007; Gianakopoulos et al., 2011; Ridgeway & Skerjanc, 2001). Indeed, these key transcription factors for muscle and neuronal differentiation were also repressed by arsenic exposure in this study, as evidenced by qPCR and immunofluorescence. Moreover, Pax7 transcripts, which have been suggested to play a key role in muscle and neuronal cells fate determination in mice (Jostes et al., 1990; Murdoch et al., 2010; Ziman et al., 2001), also is reduced by 7-fold after arsenic exposure on day 9 of embryoid body differentiation (data not shown).

In conclusion, our results indicate that 0.5μM sodium arsenite suppresses skeletal muscle and neuronal differentiation from P19 mouse embryonic stem cells due to reductions in myogenic and neurogenic transcription factors expression. The regulatory mechanism that may be responsible for these reductions after arsenic exposure is repressed Wnt/ β-catenin signaling during the early embryogenesis stage. P19 stem cells appear to be a useful model to determine the mechanisms by which toxicants impact embryogenesis.

**Acknowledgments**

This work was supported by NIH grants ES016640 and ES016640-01S1, and CSREES/USDA under project #SC-1700380. The authors would also like to thank Terri Bruce from the Clemson University Imaging Facility and Nancy Korn from Clemson University Histology Core Lab for technical assistance.

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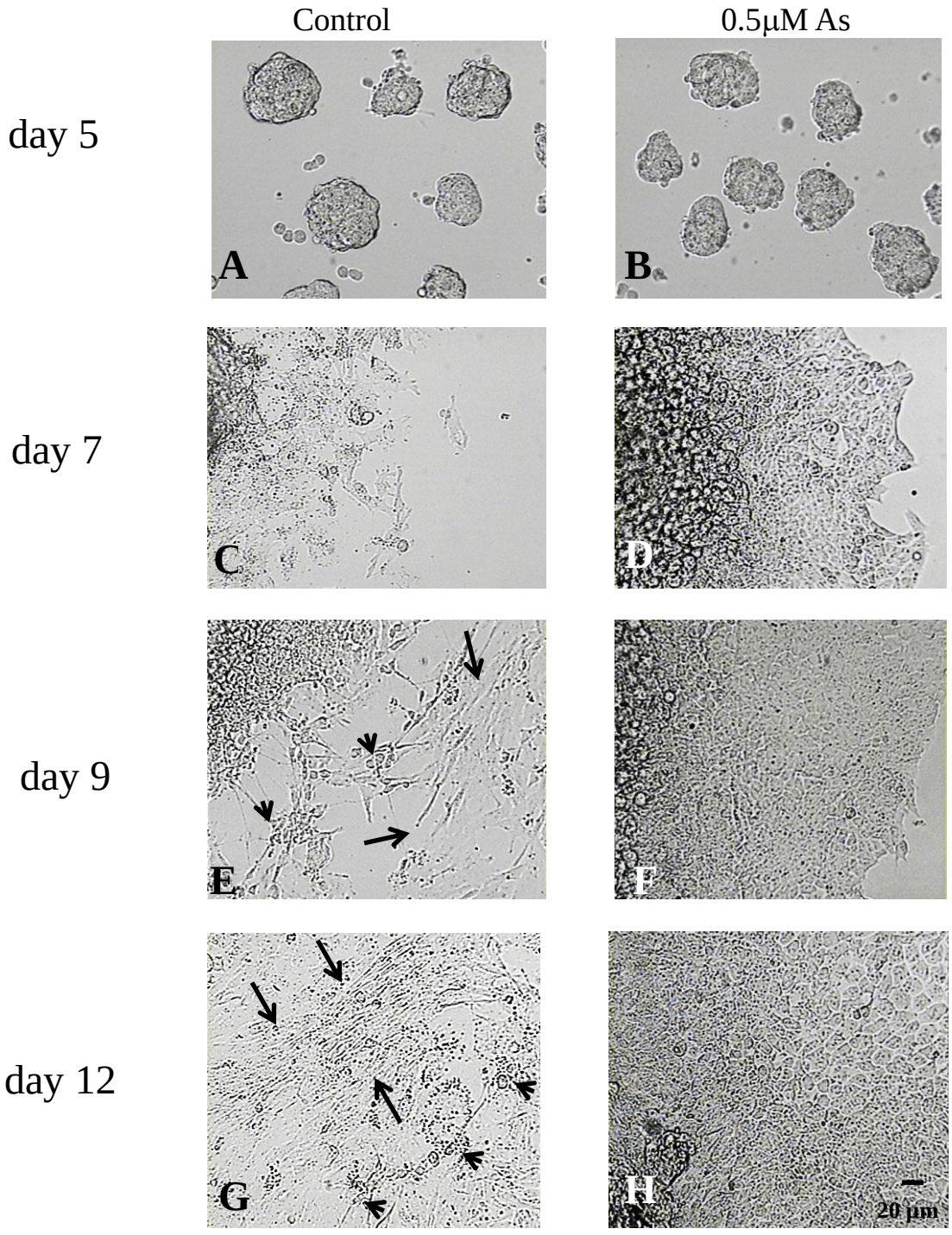


**Fig. 4.1 Arsenic represses skeletal muscles and neuronal differentiation in P19 cells.**

P19 cells were aggregated as hanging drop cultures in the presence or absence of 0.5μM sodium arsenite for 5 days (A and B), then transferred to gelatin-coated plates. On

Day 7, myoblast-like cells were observed around the embryoid bodies in the control groups (C), while on Days 9 and Day 12, myoblasts, myotubes, and neurons could be identified (E and G). Arrows show myotubes and arrowheads indicate the differentiated neurons. In arsenic-exposed groups, the P19-like cells were dominant in the outgrowth area on Days 7, 9, and 12 (D, F, and H). Pictures are representative examples from 198 independent embryoid bodies per time point per group.

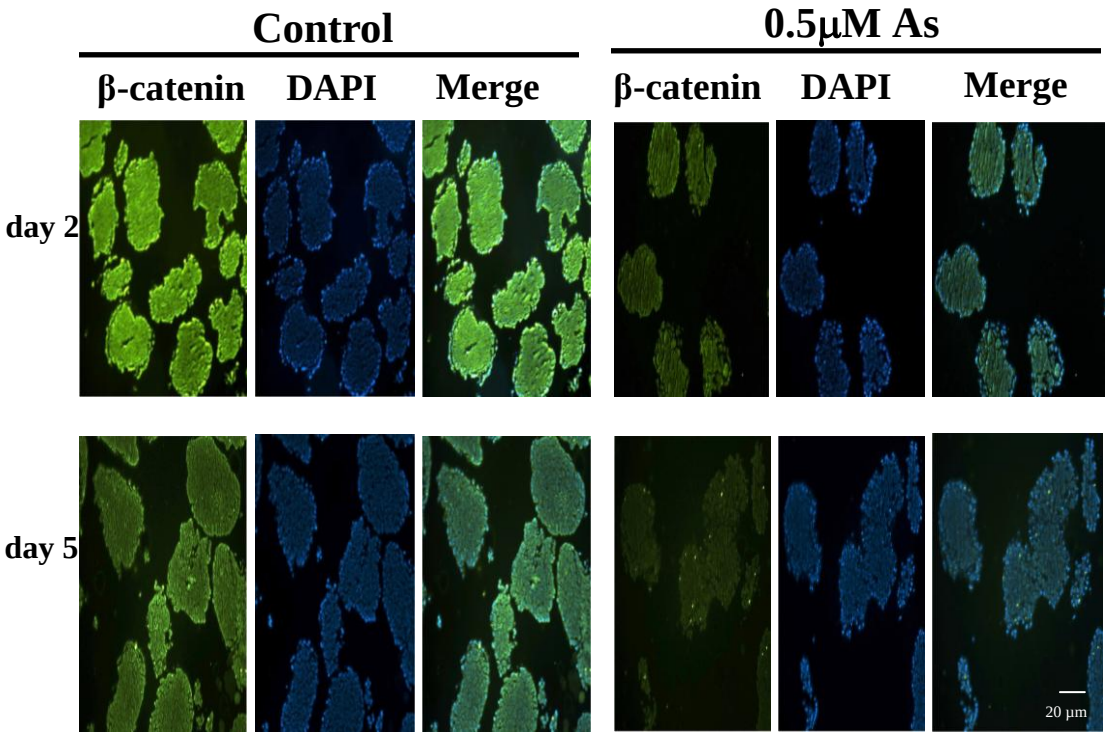


**Fig 4.2 Arsenic represses β-catenin expression in the differentiating embryoid bodies**

P19 cells were aggregated as hanging drop cultures containing 1% DMSO for 2 and 5 days with (A and C) or without (B and D) 0.5μM sodium arsenite. Embryoid bodies were harvested and embedded with paraffin, and β-catenin expression was examined by immunofluorescence. Pictures are representative examples from 198 embryoid bodies per time point per group.

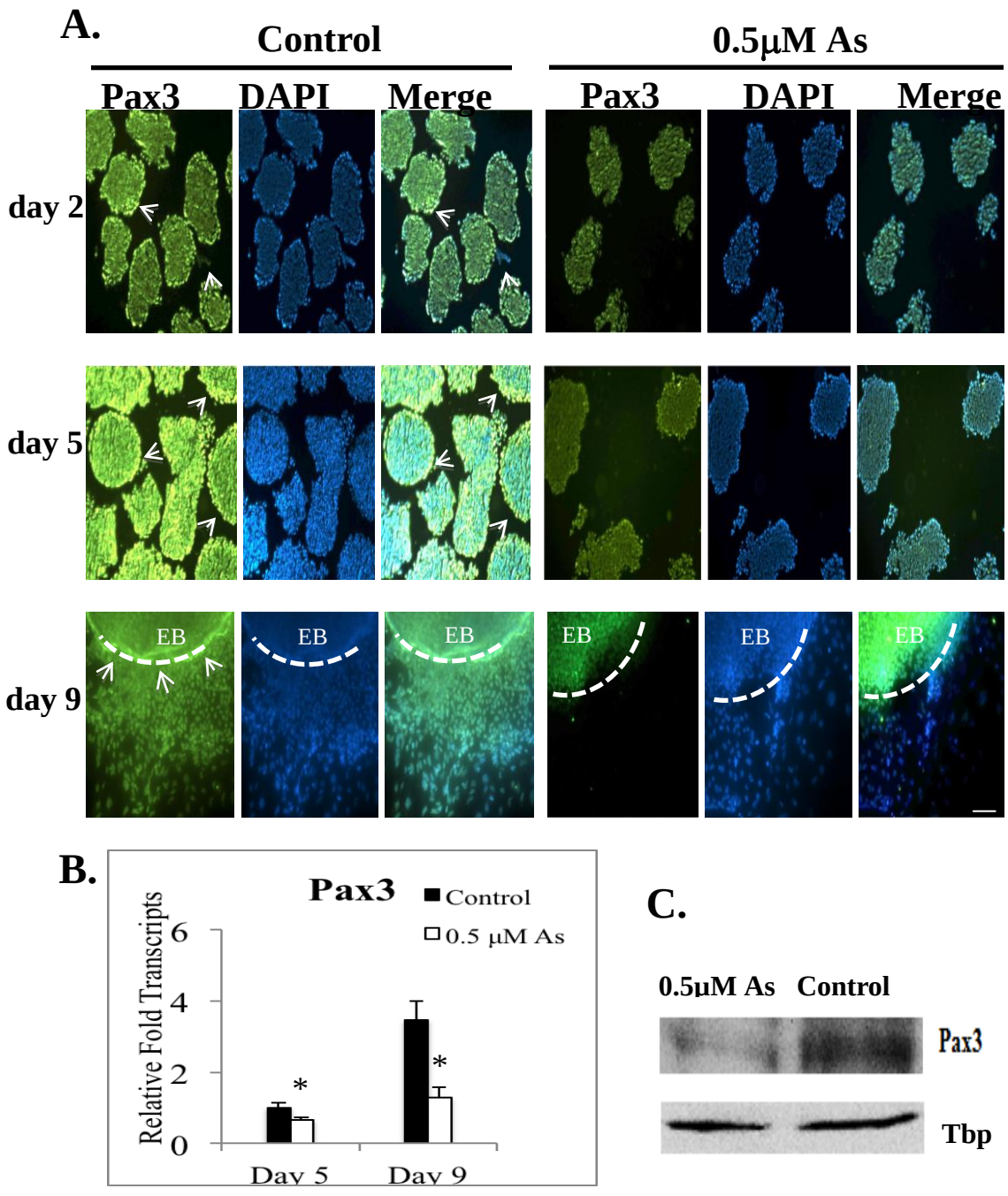


**Fig 4.3 Arsenic represses Pax 3 expression at the edges of embryoid bodies**

P19 cells were aggregated in the presence or absence of 0.5μM sodium arsenite for 5 days, then transferred to gelatin-coated plates for 4 days (Day 9) with or without 0.5μM

sodium arsenite to examine Pax3 expression by immunofluorescence (A). Arrows indicates the exterior regions of embryoid bodies with induced Pax3 expression. Dashed lines indicate the location of an embryoid body (EB). Pictures are representative examples from 198 independent embryoid bodies per time point per group. Pax3 mRNA expression on Day 5 and Day 9 was quantified by qPCR. Each sample was run in triplicate (n=3, 96 embryoid bodies/ sample/day/group) and results were normalized to GAPDH and expressed as normalized fold-change to relative to P19 5 days control cells. Statistical differences (*) were determined by Student's *t*-test ($p<0.05$) (B). Pax3 protein expression in the nuclear fraction of embryoid bodies exposed with or without 0.5μM arsenic for 5 days (Day 5) was quantified by immmunoblotting (C).

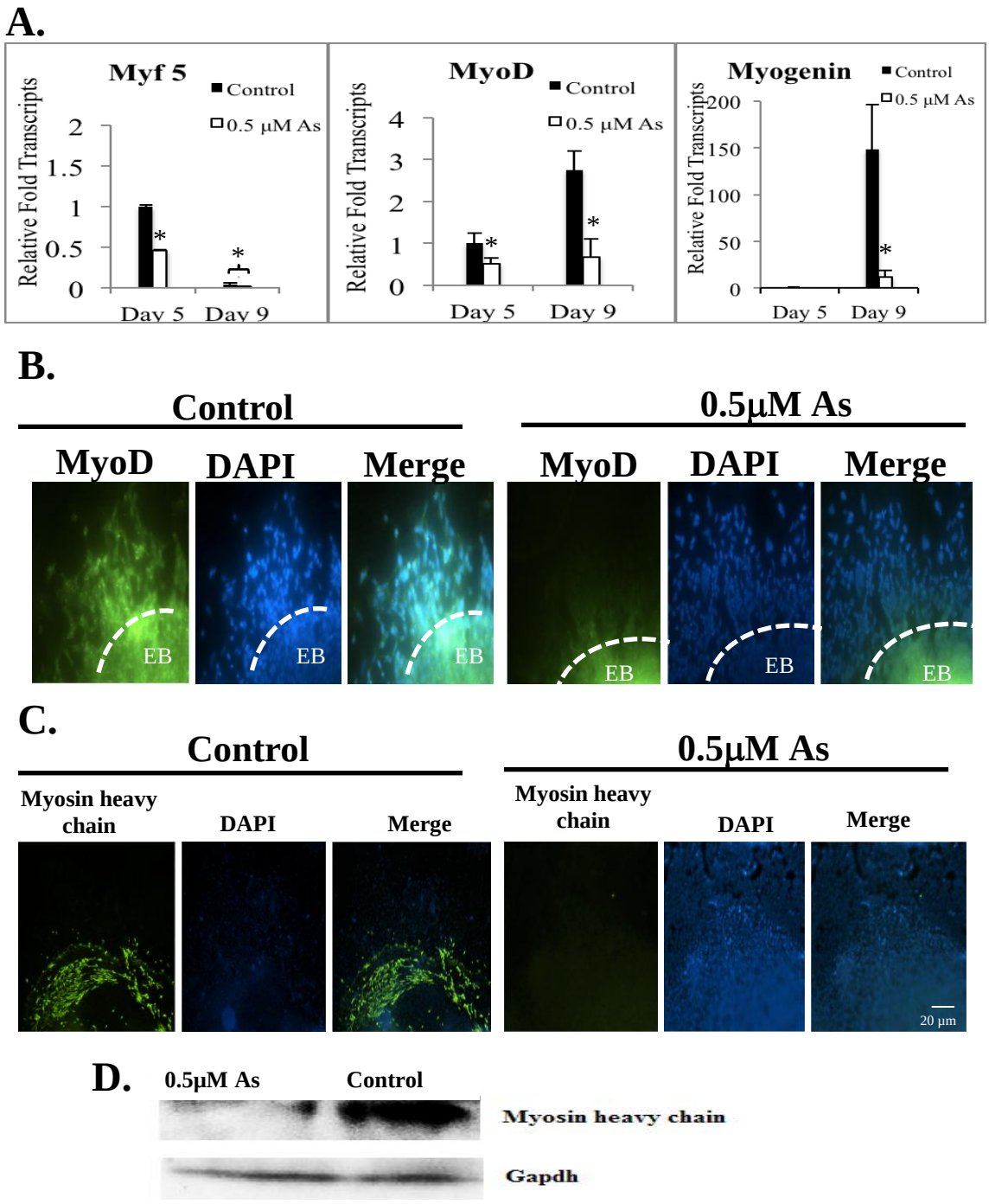


**Fig 4.4 Arsenic represses skeletal muscle differentiation through the repressed myogenic regulatory factors (MRFs)**

Myf5, MyoD, and myogenin mRNA expression in cells derived from embryoid bodies day 5 and day 9 was quantified by qPCR. Each sample was run in triplicate (n=3),

results were normalized to Gapdh, and are expressed as normalized fold-change to relative to day 5 control cells. Statistical differences (*) were determined by Student's *t*-test ($p<0.05$) (A). Differentiated cells from embryoid bodies exposed with or without 0.5μM arsenic on day 9 (B) and day 12 (C) were fixed, and MyoD (day 9) and myosin heavy chain (day 12) expression was examined by immunofluorescence. Dashed lines indicate the location of an embryoid body (EB). Pictures are representative examples from 198 independent embryoid bodies/time point/group. Myosin heavy chain protein expression was examined by immmunoblotting, using embryoid bodies exposed with or without 0.5μM arsenic on Day 12 (D).

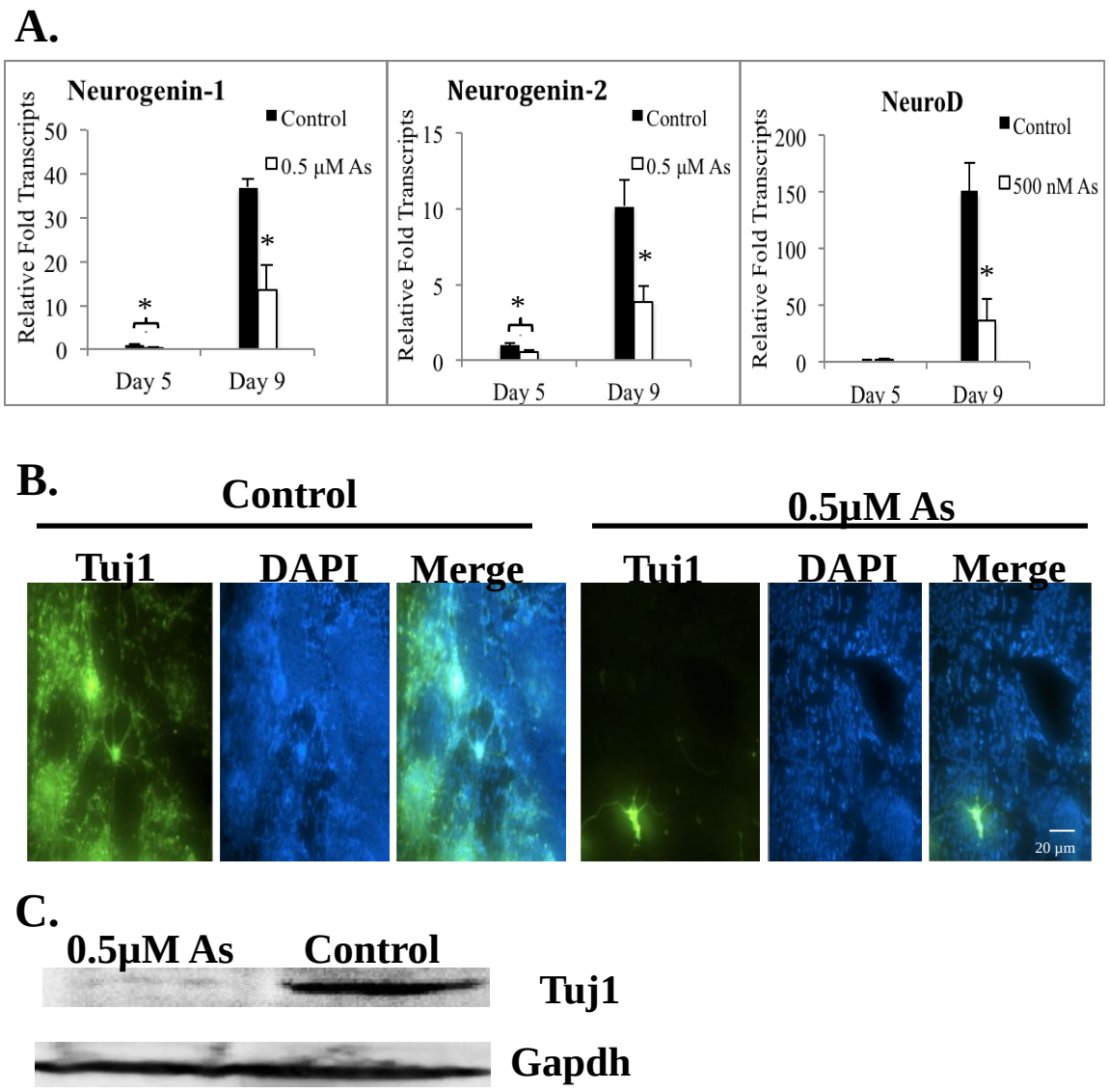


**Fig 4.5 Arsenic inhibits neuronal differentiation by repressing neurogenic transcription factors**

Neurogenin1, Neurogenin 2, and NeuroD mRNA expression from embryoid bodies exposed with or without 0.5µM arsenic for 5 days and 9 days were determined by qPCR (A). Each sample was run in triplicate (n=3), results were normalized to Gapdh, and expressed as normalized fold-change to relative to day 5 control cells. Statistical differences (*) were determined by Student's *t*-test ($p<0.05$). Differentiated control cells

and cells exposed to 0.5μM arsenite were fixed on day 12, and Tuj1 expression was examined by immunofluorescence (B). Pictures are representative examples from 198 independent embryoid bodies/time point/group. Tuj1 protein expression was quantified by immmunoblotting, using embryoid bodies exposed with or without 0.5μM arsenic on day 12 (C).

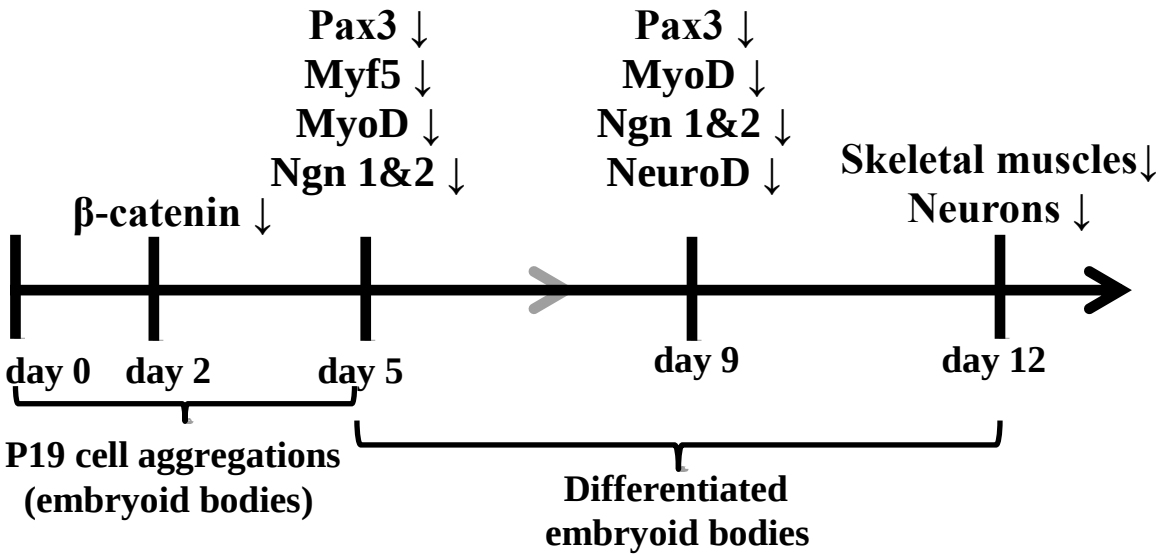


**Fig 4.6 Model of the transcription factor cascade that inhibits skeletal myotube and sensory neuron formation after arsenite exposure.**

During P19 aggregation, β-catenin expression was repressed by arsenic exposure on day 2. The repressed β-catenin leads to the reduction of Pax3, which plays an important role in specification of muscle and neuronal precursors. The reduced muscle and neuronal precursors development by arsenic exposure was supported by the repressed Myf5, MyoD, Neurogenin 1, and Neurogenin 2 expression on day 5 and day 9. The expression of myogenin and NeuroD, both could regulate terminal skeletal and neuronal differentiation (Howard, 2005; Yokoyama & Asahara, 2011), were repressed by arsenic on day 9. Thereby leading to the suppressed formation of skeletal muscles and neurons on day 12.

# CHAPTER FIVE

# CONCLUSION

The molecular mechanisms that regulate the arsenic-induced developmental toxicity are largely unclear and likely are multi-factorial. To this end, both mouse C2C12 myoblast cells and P19 stem cells were used in this study to investigate the developmental toxicity of arsenic. Our results from Chapter 2 indicate that exposure of 20 nM sodium arsenite to C2C12 myoblast cells could delay their differentiation through the reduction of myogenin expression. The repressed myogenin expression was due to altered DNA methylation patterns on the myogenin promoter and the reduced expression of myogenic transcription factor, Mef2. Chapter 3 further demonstrates that arsenic exposure to C2C12 cells could induce heterochromatin formation on the proximal myogenin promoter, reduce Igf-1 expression, and enhance the expression of muscle repressor, Ezh2. These altered mechanisms co-contribute to the reduction of myogenin, thereby leading to the delayed muscle differentiation following arsenic exposure. In chapter 4, arsenic's effects on early development were examined, using mouse P19 stem cells as a model. Our results indicate that arsenic has the ability to inhibit myogenesis and neurogenesis through the reduction of essential transcription factors in the Wnt signaling pathway.

Indeed, results from this study indicate that arsenic has the ability to silence gene expression by simply altering DNA methylation patterns on a target gene's promoter region or induce the formation of closed chromatin on critical promoter regions, where

can be interacted with the basal transcription complexes, such as RNA Pol II and TATA box binding proteins. In addition, arsenic could also repress gene expression *via* synergistically regulates the expression of growth factors (Igf-1 and myostatin), the Polycomb Ezh2 methytransferase enzyme, and the enhancer protein (Mef2). Moreover, arsenic could affect gene expression through signaling transduction. For instance, through the altered Wnt/ β-catenin signaling pathway, arsenic represses essential myogenic- and neurogenic-transcription factors, thereby leading to inhibited myogenesis and neurogenesis. Taken together, it appears that arsenic exposure could affect cellular differentiation *via* targeting cell cycles, such as the Igf-1 mediated PI3K pathway, or by epigenetic regulatory mechanisms

Data from the 2003-2008 National Health and Nutrition Examination Survey (NHANES) indicates that the median urinary arsenic concentration in the U. S. adults is 8.3 μg/L (Jones et al., 2011). Additionally, in an area of Argentina with 200ppb in drinking water, arsenic levels from placentas and cord blood from babies were 34μg/kg and 9 μg/L, respectively (Concha et al., 1998). Our results indicate that 2.7 μg/L arsenic (equivalent to a 20 ppb exposure) could delay muscle differentiation and reduce muscle fibers formation. In Mexico, school-aged children exposed to 117-1392 ppm arsenic in their drinking water have abnormal neurophysiological behavior, such as a lower IQ score and poor verbal learning and memory. Their hair arsenic levels averaged 40 ppb or 5.4 μg/kg (Caldero et al., 2001; Diazbarriga et al., 1993). Our P19 stem cell model shows that both neurogenesis and myogenesis are inhibited upon 65 μg/L arsenic exposure. Because arsenic levels in cord blood and placentas can be used as surrogates for exposure

levels for a developing embryo, which were 9 μg/L and 34 μg/kg, respectively. Our P19 cells exposed to 65 μg/L arsenic is similar to that measured in these epidemiological studies. However, there are some limitations when stem cells and attempting to compare their exposure to that of a fetus. For example, the cultured stem cells may not behave in a similar manner like inner mass cells within a fetus, that may not have the same metabolic capabilities to methylate and detoxify arsenic, and although we know what concentrations of arsenic the embryoid body was exposed to, we do not know how much is actually in the embryoid body. However, this study can help elucidate the mechanisms of how environmental realistic arsenic exposure impacts early development.

Arsenic is present in water systems around the world, but the mechanisms by which arsenic causes health effects are still not well understood. Because epidemiological studies indicate that long-term exposure to arsenic can result in cancer and other adverse outcomes in skeletal and neuronal development (Fig 1.1 and 1.2), the United States Environmental Protection Agency (EPA) reduced the allowable concentration of arsenic in drinking water from 50 ppb to 10 ppb by 2006 (EPA, 2001). However, the tighter standards have been questioned, because most of the epidemiological studies were conducted in Bangladesh, Chile, Taiwan, and India, where the arsenic concentrations are generally higher than that in the United States (Chen et al., 1988; Rahan et al., 2007; Smith et al., 1998). This study supports the EPA's tighter standards about the regulations of arsenic in public drinking water systems. This study demonstrates that exposure of 20nM arsenic or 2.6ppb arsenic, which is below current drinking water standard of 10ppb, can result in the delayed muscle differentiation and lead to improper organization

of muscle fibers. Since improper organized muscle fibers may ultimately cause abnormal physiological outcomes on organism, it is reasonable to ask whether the drinking water standard for arsenic is protective of public health, especially for the aspects of fetal development. To this end, Canada has reevaluated arsenic concentration in drinking water. The proposed allowable arsenic in drinking water is 5 ppb (Kapaj et al., 2006).

To date, the risk assessment of arsenic exposure at low levels is limited, due to a lack of data (Gibb et al., 2011). To extrapolate risk of low-dose arsenic exposure by using traditional whole-animal experiments is sometimes not feasible. For example, animals are often treated with high tolerable doses so that hazards can be identified (van Vliet, 2011). Such high concentrations are very different from real-life environments, because humans are exposed to low levels of environmental chemicals, such as arsenic in drinking water. Therefore, a mouse P19 stem cell line was used in this study to evaluate the impacts of low-level arsenic exposure on cellular differentiation. Results from 500 nM arsenic exposure indicate arsenic inhibits myogenesis and neurogenesis through the repressed essential myogenic- and neurogenic-transcription factors, caused by the reduction of Wnt/ β-catenin signaling pathway during early embryogenesis. To our knowledge, this is the first report that uses stem cell-based assays to examine the adverse developmental effects of low-level arsenic exposure in the embryogenesis. This arsenic-exposed P19 stem model may, at least in part, give us insight into what has been observed from the epidemiological studies that show the arsenic-mediated adverse developmental effects during pregnancy. Most importantly, by combination of other approaches, such as -omics analysis, assessments of cytotoxic effects on undifferentiated/differentiated P19 cells, and

$IC_{50}$ analysis of three germ layers, this P19 model has potential for the comprehensive evaluation of arsenic-mediated developmental toxicity in the future.

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